Pyoverdine Production and its Social Effects in Natural Communities of *Pseudomonas* Bacteria

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Dedicated to my parents,

especially to the memory of my wonderful mother, Olga Butas,

who always believed in me, immensely loved and supported me,

and whose selfless kindness and love to others will always inspire me.

‘Somewhere, something incredible is waiting to be known.’

~ Sharon Begley (1977)

Contents

Summary

Iron is a key growth-limiting factor for most bacteria. It is required for various essential cellular processes, like DNA biosynthesis and respiration. However, in nature iron is generally limited. To overcome iron limitation, many bacteria secrete siderophores, iron-scavenging molecules that have a high affinity for ferric iron. These molecules bind iron from the surrounding environment and are taken up by cells via specific membrane-embedded receptors. Siderophores are not only needed to acquire iron but also serve in competition with others: the secreted siderophores can lock iron away from competitors with incompatible receptors. Moreover, siderophores can be shared among individuals with compatible receptors. While cooperative siderophore sharing might be a beneficial strategy, it can also select for siderophore-negative strains with one or many receptors. Such non-producers can act as cheaters exploiting siderophores of others, which benefits cheaters but reduces the fitness of producers. Siderophore production and siderophore-mediated interactions have been extensively studied in laboratory conditions. However, we know only little about the determinants of siderophore production and their sociality in the complex bacterial communities in nature. The aim of my thesis was to bridge this gap in knowledge.

I carried out two main projects: (i) project 1 mainly focuses on siderophore cooperation and cheating, and (ii) project 2 focuses on the environmental determinants of siderophore production and siderophore-mediated interactions. For this, I first isolated 930 *Pseudomonas* strains from 48 different soil and pond communities. I was interested in their main, high-affinity siderophore pyoverdine, whose structure can vary between different pseudomonads. Pyoverdine production has been extensively studied in laboratory conditions (especially in the opportunistic human pathogen *P. aeruginosa*) and has become a model example of bacterial cooperation.

In the first project, using 315 isolates, I focused on pyoverdine production to study its exploitability among community members, the fitness consequences of the ability or inability to exploit heterologous pyoverdines, and the genetic basis of the observed pyoverdine-mediated interactions. I found that pyoverdine non-producers occur in many soil and pond communities. Some non-producers could act as cheaters on the producers secreting a compatible pyoverdine. Conversely, non-producers could also be inhibited by structurally different pyoverdines from other community members. The results of this project suggest that in nature there is both selection for cheating and resistance against cheating, which could drive antagonistic co-evolution and diversification in bacterial communities.

In the second project, using all 930 isolates, I studied how different environmental determinants (habitat structuring, pH, total iron and carbon concentrations, community diversity) could be involved in shaping pyoverdine production and its social consequences (i.e. ability to be exploited and its inhibitory effect on non-producers) in natural *Pseudomonas* communities. These factors were shown

in laboratory conditions to affect level of pyoverdine production and success of cheating non-producers. My results suggest that the considered factors are also important in nature. However, contrary to the well-controlled laboratory conditions, in nature these factors turned out to be often positively or negatively correlated with each other. Consequently, combinatorial or trade-off effects between these factors often seemed more important in determining patterns of pyoverdine production and effect on non-producers than a single factor alone. I also observed clear differences among pond and soil. For example, pond isolates produced on average more pyoverdine than soil isolates. Furthermore, the frequency of non-producers was highest in soils with predicted high bioavailability of iron. This suggests that the loss/absence of pyoverdine production can be favoured not only because of the opportunity to cheat, but also because pyoverdine might be not necessary for growth in some habitats.

Taken together, I uncovered how bacteria from natural communities behave and interact with each other under iron limitation, and how this is related to their ecology and genetic background. These findings allow to infer co-evolutionary patterns, determine drivers of diversification and possible consequences for the stability of natural communities. Moreover, my findings can be interesting for applied research, as siderophore production is also relevant for inhibition of pathogens with less efficient iron acquisition strategies, heavy-metal detoxification and virulence. It has been shown that the evolution of siderophore non-producers can significantly disturb these processes. Thus, my findings can be useful for those who seek to suppress the spread of non-producers in siderophore-based plant-pathogen control, bioremediation processes, or aim to develop therapeutic approaches promoting the spread of the less virulent non-producers in infections.

Zusammenfassung

Eisen ist ein wichtiger Wachstumssfaktor für die meisten Bakterien. Es wird für verschiedene essentielle zelluläre Prozesse gebraucht, wie z.B. die DNA-Biosynthese und Atmung. Allerdings ist in der Natur Eisen in der Regel nur sehr begrenzt verfügbar. Um die Eisenlimitierung zu überwinden, sekretieren viele Bakterien Siderophore, eisenbindende Moleküle, die eine hohe Affinität für Eisen haben. Diese Moleküle binden Eisen aus der Umgebung und werden von Zellen über spezifische eingebettete Membranrezeptoren aufgenommen. Siderophore sind nicht nur nötig, um Eisen aufzunehmen, sondern können auch für das erfolgreiche konkurrieren gegenüber anderen Bakterienarten eingesetzt werden. Die ausgeschiedenen Siderophore können zum Beispiel die Verfügbarkeit von Eisen für Konkurrenten mit inkompatiblen Rezeptoren verringern. Siderophore können aber auch zwischen Bakterien mit kompatiblen Rezeptoren geteilt werden. Kooperatives Teilen von Siderophoren kann einen Vorteil für die ganze Population bringen, macht es aber auch anfällig für Individuen, die dieses System ausnutzen. Bakterien, die keine Siderophore produzieren, können als Betrüger wirken indem sie die Siderophore von anderen ausnutzen, und damit die biologische Fitness der Produzenten reduzieren. Siderophor-Produktion und die damit zusammenhängenden Interaktionen wurden bisher intensiv unter Laborbedingungen untersucht. Allerdings wissen wir nur wenig über die Faktoren, welche die Siderophorproduktion und ihre Sozialität in den komplexen bakteriellen Gemeinschaften in der Natur beeinflussen. Das Ziel meiner Arbeit war es, diese Wissenslücke zu überbrücken.

Ich habe zwei Hauptprojekte durchgeführt: (i) Das Projekt 1 konzentrierte sich vor allem auf die Siderophor-Kooperation und das ausnützen des Systems. (ii) Projekt 2 konzentrierte sich auf die ökologischen Determinanten der Siderophorproduktion und der siderophorbasierenden Interaktionen. Dafür hatte ich zuerst 930 *Pseudomonas*-Stämme aus 48 verschiedenen Boden- und Teichgemeinschaften isoliert. Der Fokus meiner Studien lag auf dem wichtigsten Siderophor von *Pseudomonas*, dem hochaffinen Pyoverdin, dessen Struktur zwischen verschiedenen Pseudomonaden stark variieren kann. Die Pyoverdinproduktion wurde intensiv unter Laborbedingungen (insbesondere im opportunistischen menschlichen Pathogen *P. aeruginosa*) untersucht und ist zu einem Modellbeispiel für bakterielle Kooperation geworden.

Im ersten Projekt, mit 315 Isolaten, konzentrierte ich mich auf die Produktion von Pyoverdin, um die Teilbarkeit dieses Moleküls zwischen verschiedenen Isolaten zu untersuchen. Insbesondere interessierte ich mich dafür, welche Fitnesskonsequenzen die Fähigkeit fremdes Pyoverdin auszunutzen hat und welche genetischen Faktoren diese Fähigkeit beeinflussen. Ich fand, dass Stämme, die kein Pyoverdin produzieren, in vielen Boden- und Teichpopulationen vorkommen. Zudem konnte ich zeigen, dass einige Nicht-Produzenten fähig waren diejenigen Produzenten auszunutzen, welche ein kompatibles Pyoverdin sekretieren. Umgekehrt wurden aber genau dieselben

Nicht-Produzenten in ihrem Wachstum gehemmt, wenn sie mit einem strukturell unterschiedliche Pyoverdine konfrontiert wurden. Diese Ergebnisse deuten darauf hin, dass die natürliche Selektion sowohl das Ausnutzen als auch Strategien gegen das ausgenutzt werden fördert. Dies könnte antagonistische Co-Evolution und Diversifizierung in bakteriellen Gemeinschaften vorantreiben.

Im zweiten Projekt, bei dem alle 930 Isolate verwendet wurden, untersuchte ich, wie unterschiedliche Umweltfaktoren (Habitatstrukturierung, pH-Wert, Gesamteisen- und Kohlenstoffkonzentrationen, gemeinschaftliche Vielfalt) die Pyoverdinproduktion beeinflussen könnten, und wie sich diese Faktoren auf die sozialen Interaktionen (Ausnutzung vs. Hemmung) innerhalb der natürlichen Pseudomonasgemeinschaften auswirken könnten. Meine Ergebnisse deuten darauf hin, dass im Gegensatz zu den gut kontrollierten Laborbedingungen, diese Faktoren in der Natur komplexere Rollen einnehmen und oft positiv oder negativ miteinander verknüpft sind. Folglich schienen kombinatorische oder gegenläufige Effekte zwischen diesen Faktoren oft bestimmender zu sein, bezüglich dem Umfang der Pyoverdinproduktion und deren Auswirkung auf Nicht-Produzenten, als ein einzelner Faktor allein.

Ich habe auch deutliche Unterschiede zwischen Teich- und Bodenhabitaten beobachtet. Zum Beispiel produzierten Teichisolate im Durchschnitt mehr Pyoverdin als Bodenisolate. Darüber hinaus war die Häufigkeit der Nichtproduzenten am höchsten in Böden mit vorhergesagter hoher Bioverfügbarkeit von Eisen. Dies deutet darauf hin, dass der Verlust / die Abwesenheit der Pyoverdinproduktion nicht nur durch die Verfügbarkeit von fremden Siderophoren abhängt, sondern auch dadurch, dass man nicht auf die Produktion angewiesen ist.

Zusammenfassend lässt sich sagen, dass meine Dissertation einen wichtigen Beitrag leistet, um zu verstehen, wie sich Bakterien aus natürlichen Gemeinschaften unter Eisenlimitierung verhalten und miteinander interagieren und wie dies mit ihrer Ökologie und ihrem genetischen Hintergrund zusammenhängt. Diese Erkenntnisse erlauben es, gemeinsame evolutionäre Muster zu ermitteln, und die treibenden Kräfte hinter der biologischen Diversifizierung und ihrer Konsequenzen für die Stabilität von natürlichen Gemeinschaften besser zu verstehen. Darüber hinaus können meine Erkenntnisse für die angewandte Forschung interessant sein, da die Siderophor-Produktion auch für die Hemmung von Pathogenen wichtig ist, und Siderophore für die Entgiftung von schwermetall-verseuchten Böden verwendet werden können. In beiden Beispielen wurde gezeigt, dass die Evolution von Stämmen, welche keine Siderophore mehr produzieren können, eine wichtige Rolle spielen.

Chapter 1. Introduction

‘“What a big book, captain, might be made with all that is known!”

“And what a much bigger book still with all that is not known!”’

~ Jules Verne, The Mysterious Island (1874)

1.1 The struggle for iron

1.1.1 Iron limitation

Iron is the fourth most common element in the Earth's crust. It is required by almost all organisms [1] for different cellular processes, like DNA replication, photosynthesis, respiration and nitrogen fixation. It exists mainly in two oxidation states, as ferric (Fe^{3+}; prevails in aerobic conditions; usually found as highly insoluble oxides) and the highly soluble ferrous (Fe^{2+}; prevails in anaerobic and low-pH conditions) forms. It was suggested that iron became a part of many important proteins early in the evolution of life, because of its chemical versatility and its high general bioavailability (there was still no oxygen, and thus availability of iron was high). However, when photosynthesis occurred and levels of oxygen started to increase, iron became less and less available [2]. Moreover, iron became toxic when present in high quantities, as in aerobic conditions it forms reactive oxygen species (via Fenton reaction) [3]. Therefore, the previously anaerobic organisms had to adapt by evolving tight regulation of iron acquisition and storage. Furthermore, some organisms had to adapt when they became associated with hosts where iron is bound to host proteins, and is thus limited.

Iron is usually limited in anaerobic, circumneutral environments and in a host environment [2,4]. The very poor iron availability in oceans is the main factor restricting phytoplankton growth even in areas rich in macro-nutrients (high-nutrient, low chlorophyll regions) [5,6]. Poor iron availability is also posing a problem, for example, to plant growth in alkaline aerated soils [7]. pH is thought to be one of the main factors affecting solubility of iron, and other metals, in aerobic soil and aquatic systems (its solubility decreases with increasing pH) [4,8,9]. Moreover, other factors, like organic matter [9–12], sunlight [4] and water content [7,13] may play an important role in making iron more bioavailable.

Bacteria possess different mechanisms to cope with iron limitation, tightly regulated by the iron availability in the environment [2,6,14]. To acquire iron, (i) many of them secrete ferric-iron-chelating siderophores; they can also (ii) secrete or have surface reductases that reduce ferric iron to the more soluble ferrous iron; (iii) acidify their local environment making iron more soluble; (iv) use iron chelators of the host; (v) or take up iron-binding organic acids, like citrate. Moreover, bacteria can store iron in ferritins, bacterioferritins and/or DNA-binding Dps proteins. These proteins serve two functions: to supply iron when environmental iron is scarce, and to protect cells from iron toxicity (for example, Dps protects DNA from oxidative stress generated via Fenton reaction) [2,15].

1.1.2 Siderophores

Siderophores are low molecular weight iron-binding compounds (0.5 – 1.5 kDa) with very high affinity for ferric iron, and recognized by specific membrane-embedded receptors. They are common among bacteria, fungi and graminaceous plants [14], and play a very important role not only in iron

acquisition but also, looking more broadly, in mineral weathering in nature, significantly increasing solubility of iron [16]. There are more than 500 different microbial siderophores known [6]. Generally, siderophores and their receptors are specific. However, also less specific receptors and/or several different receptors in the same strain can be present, enabling acquisition of siderophores from members of the same or different genera [17,18]. This use of heterologous siderophores, also called 'siderophore piracy' [19], is expected to confer a selective advantage in competition for iron [20]. Additionally, siderophores can serve to inhibit competitors [21–24] that have less efficient iron acquisition strategies, i.e. siderophores with lower affinity for iron or siderophore non-producers, with no receptors for heterologous siderophores [20]. The inhibition occurs because siderophores further reduce iron availability to the competitors [21,25], for iron and/or other resources. Thus, presence of receptors allowing acquisition of these siderophores would protect bacteria from such inhibition [24], and could also help to invade new niches occupied by siderophore producers [23].

A few studies on laboratory and natural bacterial strains demonstrated that different abiotic or biotic factors, usually related to iron availability, can affect the level of siderophore production. For example, some pseudomonads were shown to downscale siderophore production in response to higher iron concentration [26], or increased iron solubility at lower pH [27], and when iron was bound to relatively weak organic chelators [28]. Siderophore production can also be up- or downregulated in the presence of other heavy metals, that compete with iron for the binding sites of siderophores, and/or directly affect regulation of siderophore production [29]. Interestingly, bacteria can often produce more than one type of siderophore. These siderophores can differ in the conditions at which they operate optimally (e.g. different pH; different degree of iron limitation) [28,30,31], which can be related to the difference in their production cost and affinity for iron. Such diversity is suggested to reflect adaptation to fluctuating environments. For example, when iron is less limited, *Pseudomonas aeruginosa* produces more of the siderophore pyochelin, which is less costly and has much lower affinity for iron than the siderophore pyoverdine. Conversely, production of the costlier pyoverdine is upregulated when iron is very limited [28]. Astonishingly, *Escherichia coli* can have up to four different siderophores, with different pH optima, which might be especially useful when colonizing environments with fluctuating pH, like urine [31]. Level of siderophore production can also be affected by the presence of other species. For example, some bacteria downregulate their siderophore production in the presence of another species, whose siderophore they can use [32], or increase production if they are not able to use the siderophore of their competitor [33–35]. Interestingly, production of siderophores of some isolates appearing as non-producers can be induced by heterologous siderophores [36].

1.1.3 Siderophore-independent iron-acquisition systems

Apart from siderophores, bacteria also have other means to acquire iron. For example, they can secrete reductases, reducing the insoluble ferric iron to the readily available ferrous iron [37], or hemophores to

take up heme of the host [38,39]. However, the secreted compounds might be lost, depending on the environment where they are secreted. For example, they might: (i) diffuse away, especially in highly-diluted well-mixed environment like oceans; (ii) be strongly adsorbed to surfaces, e.g. of minerals; (iii) be taken up by others; or (iv) be degraded. Therefore, big amounts of these compounds might be needed in order to receive benefits of their secretion [40], but costs of their production might be higher than benefits. In this case (e.g. when siderophore diffusion is not limited) bacteria could employ more private strategies. Indeed, along with siderophore production, bacteria often possess iron-acquisition systems associated with a membrane, thus, their loss to the abiotic and biotic environment is avoided. Such systems include the widespread Feo receptors for ferrous iron [2], ferric iron transporters [41,42], receptors for host proteins (e.g. transferrin, lactoferrin) [43], surface reductases [44], and ferric citrate receptors [45,46].

Given that siderophores are costly to produce and might be lost, why do then most bacteria have siderophore systems? Why do they not simply use their membrane-embedded systems? Secreted compounds can be public, thus, they can be more interactive than the membrane-associated ones, i.e. they can more positively or negatively affect others in the local environment. For example, siderophores can inhibit competitors from a distance, without engaging in a possibly deadly cell-to-cell contact. Moreover, it was predicted that siderophore secretion can be an efficient strategy even in a very diluted environment, such as the ocean, provided their production is a cooperative behaviour of many cells staying close [40]. Such cooperative siderophore secretion is expected to significantly increase solubility of large insoluble iron aggregates [47], which could not be achieved if they were kept private.

1.2 Cooperation and cheating in microbes

Some siderophores were shown to be public goods, i.e. sharable secreted molecules, which are costly to produce but provide benefits to the local group [48]. Production of such sharable siderophores is a well-studied example of cooperative behaviour among bacteria. Cooperation is a behaviour that benefits the recipient of a cooperative act and is selected for, at least partially, because of this beneficial effect [49]. It can be further divided into altruistic behaviour, which is costly to the actor but beneficial to the recipient; and mutually beneficial behaviour, which is beneficial for both the actor and the recipient [48]. When siderophores are released into environment and bind iron, they can also be taken up by other members of the community that have a compatible receptor. Such sharing can be mutual, but cells can also get back less than what they invested, thus, siderophore production might also be an altruistic behaviour.

Microbes can engage in many other social interactions, like fruiting body formation [50], cell-to-cell communication via signalling molecules (quorum-sensing) [51], degradation of antibiotics [52], biofilm

formation [53] or social motility through the secretion of biosurfactants [54]. Existence of bacterial cooperation is puzzling, as it is predicted to be invaded by social cheaters, cells that do not cooperate or cooperate less but exploit cooperation of others [49]. Such exploitation increases reproductive success of a cheater, but reduces the reproductive success of the cheated individual [55,56], which results in relative fitness advantage of the cheater over the cooperator [57]. Such behaviour can arise both within and among species [56].

Social cheating has been demonstrated for different traits and different microbial laboratory systems, for example, for siderophore production, where siderophore non-producers have a siderophore receptor and exploit siderophores produced by cooperators [33,58], fruiting body formation [50], swarming motility, quorum sensing and biofilm formation [59,60]. Different studies, which mostly evolved bacteria under laboratory conditions, showed that there are various factors preventing social cheaters from driving cooperators to extinction. For example, environmental factors like structuring can limit access of cheaters to the cooperators and their public goods [61]. Furthermore, cooperators can evolve more private 'public' goods, e.g. aquatic bacteria produce siderophores with hydrophobic parts that tend to aggregate and stick to the producer [62–64]; *E. coli* siderophore enterochelin is kept private at low cell density [65]. Moreover, cooperators could recognize other cooperating relatives [66], protecting themselves from cheaters [67]. In addition, a gene for a social trait might be linked to other important traits, therefore, its loss might be disadvantageous [68]. Furthermore, expression of a cooperative trait might be regained, e.g. via horizontal transfer of genes for cooperative trait [69,70]. Selection for siderophore-negative cheaters was shown to be lower with increased iron [26,33] or carbon [71,72] availability, as it reduces the cost of siderophore production. Thus, bacteria can resist cheating also by metabolic prudence: e.g. *P. aeruginosa* produces and secretes a public good biosurfactant (required for social motility) only when metabolic cost of its production is low [54]. Finally, antagonistic behaviours specifically suppressing cheaters can also occur [73,74].

Strains with different levels or no expression of a cooperative trait (e.g. siderophore, fruiting body formation, swarming motility) were found to evolve not only in laboratory conditions but also in different natural environments [70,75–80]. Such strains are often automatically considered cheaters. However, Kraemer and Velicer (2014) [57] showed that even if soil *Myxococcus xanthus* strains that are relatively bad in social swarming and sporulation could increase in fitness when present with a strain with higher expression of a cooperative trait, this was not enough to gain a relative fitness advantage over the cooperators. Thus, non- or low cooperative phenotypes are not necessarily true cheaters. Moreover, the same strain can be able or not to cheat depending on the context [81,82].

1.3 Siderophore non-producers in nature

Siderophore non-producers were found in different natural environments, like oceans and human lungs infected by the opportunistic pathogen *P. aeruginosa* [70,76,78,80]. It is still debated what fosters their evolution and maintenance. Currently, there are three main hypotheses to explain the existence of siderophore non-producers: (i) cheating behaviour, (ii) evolution of dependencies, and (iii) trait loss because of disuse. There are a number of studies on natural communities that support the cheating hypothesis: the *Vibrio* non-producers were shown to lack siderophore biosynthesis genes [70], whereas *P. aeruginosa* non-producers often have mutations in the regulator of siderophore biosynthesis [76]. Compatible with the idea of cheating, these strains retained a receptor for siderophore uptake [70,76], thus, have a potential to exploit siderophores produced by others. There is also evidence for the evolution of dependencies. Interestingly, many bacteria can be cultured only in the presence of heterologous siderophores [19]. It was suggested that the low culturability of bacteria from natural environments (less than 1% of bacterial populations) [83] can often be due to the dependency of these bacteria on molecules produced by others [19]. A recent theory, termed 'Black Queen hypothesis' [84], predicts that such dependencies evolve when some community members lose a vital function because they can continuously use 'leaky' (partially available to others) functions performed by other community members, leading to their genome reduction. In this context, the fitness of producers is not necessarily reduced. Furthermore, such dependency might further evolve into a mutualistic interaction, where a non-producer expresses other traits that benefit the producer lacking the trait [57,85]. However, it is not known whether the environmental siderophore non-producers can cheat on producers, are inhibited in their presence or have other interactions, such as mutualism. Finally, there is also some evidence for the disuse hypothesis: siderophore production was shown to be lost when an alternative iron acquisition system (heme uptake) exists [86]. Moreover, as hypothesized but not tested, siderophore production might be lost because iron is readily available in the habitat [56]. Given the deletional bias of bacterial genomes [87], this loss of function should lead to the loss of genes related to biosynthesis of the siderophore and its uptake [76], unless products of these genes serve other functions than iron acquisition [24].

1.4 Bacteria and siderophores studied in this thesis

Since this thesis focuses on siderophore production and siderophore-mediated social interactions among fluorescent pseudomonads, I briefly introduce here these bacteria and their main siderophore.

1.4.1 Fluorescent pseudomonads and pyoverdine

Pseudomonas are rod-shaped γ-proteobacteria that are abundant in nature, versatile and well adapted to different environments, such as soil, aquatic systems, plants and animals. Some of them are

pathogens, e.g. *P. aeruginosa* and *P. syringae*, human and plant pathogens, respectively. Strains promoting plant growth and inhibiting plant pests were also identified [88,89]. Fluorescent pseudomonads produce a high-affinity siderophore pyoverdine that fluoresces under UV light. Like other siderophores, it can also bind other metals than iron (e.g. Co^{2+} , Cu^{2+} , Ga^{3+} , Zn^{2+}), albeit with lower affinity than ferric iron (except for Ga^{3+}). Furthermore, such pyoverdine-metal complexes, at least in *P. aeruginosa*, cannot enter or enter cells with low efficiency. This is because the receptors are specific to ferripyoverdine (ferric iron-bound pyoverdine) [90]. Additionally, many pseudomonads can produce a second, less costly siderophore with lower affinity for iron, like pyochelin, thioquinolobactin, yersiniabactin, corrugatin and ornicorrugatin [91].

Pyoverdine production has been extensively studied in the laboratory as a model of cooperative behaviour involving public goods. Laboratory experiments have shown that pyoverdine is a public good that can be shared among cells, and be exploited by cheating mutants, in the context of iron acquisition [92] and heavy metal bioremediation [93]. Because of its ability to bind different heavy metals and low affinity of pyoverdine receptors for non-iron complexes of pyoverdine, the siderophore could be used in heavy metal bioremediation. However, Cu^{2+} toxicity was shown to select for pyoverdine-negative cheaters even more than iron limitation [93]. Such cheaters, although not paying cost of pyoverdine production, benefit from the Cu^{2+} detoxifying effect of pyoverdines secreted by producers. Therefore, in order for the bioremediation to be successful, conditions disfavouring spread of cheaters/non-producers should be created. Pyoverdine was shown to be also involved in virulence [94,95], thus, pyoverdine non-producers are less virulent than producers. This can be used for the development of novel therapeutic approaches that favour spread of non-producers [96].

1.4.2 Pyoverdine production and diversity

Pyoverdine is a secondary metabolite produced via non-ribosomal peptide synthesis, in which more than 12 enzymes are involved. It is recognized and taken up via a cognate TonB-dependent receptor embedded in the outer membrane, called ferripyoverdine receptor [97]. Pyoverdine consists of a conserved chromophore (making pyoverdine naturally fluorescent), an acyl side chain linked to the chromophore, and a variable peptide chain (6 - 12 amino acids). The different *Pseudomonas* strains often produce slightly different pyoverdines, that vary in the length and the composition of the peptide chain [98,99]. It was shown that in *P. aeruginosa* pyoverdine biosynthesis genes and receptor are under diversifying selection [100]. FpvA (one of the ferripyoverdine receptors found in *P. aeruginosa*) has been suggested to drive diversity at the *pvd* locus (a region that contains *fpvA* and most of the other genes involved in synthesis and transport of pyoverdine) [100,101]. As ferripyoverdine receptors are common entry points for pyocins (a type of bacteriocins) and phages [102,103], receptor diversity was proposed to serve as a resistance mechanism [100]. Alternatively, the high pyoverdine diversity (more than 100 different pyoverdines are known [104]) was hypothesized to be driven by antagonistic co-evolution

between a pyoverdine producer and its cheater. It could serve as a mechanism to avoid exploitation by cheaters, as changes in pyoverdine structure, followed by changes in the uptake receptor, would make it more exclusive to the producer and less accessible to cheaters [100,105].

Pyoverdine production and uptake are tightly regulated in response to iron limitation [28,106]. In *P. aeruginosa* pyoverdine was shown to regulate its own production. This regulation involves the iron starvation extracytoplasmic sigma factors (ECF) PvdS and FpvI, the pyoverdine receptor FpvA, and the anti-sigma factor FpvR. First, the ferric iron-bound pyoverdine interacts with its receptor FpvA, which leads to FpvR degradation. This, in turn, activates PvdS and FpvI, which upregulate biosynthesis of pyoverdine and its receptor, respectively [107]. When enough iron is taken up, pyoverdine biosynthesis is silenced via the iron-binding transcriptional repressor Fur (ferric uptake regulator): the iron-bound Fur represses the promoter of *pvdS*, which encodes the PvdS [108]. Not only FpvA, but many TonB-dependent receptors in pseudomonads seem to be tightly regulated by ECFs, and expressed only when cognate or compatible heterologous siderophores are present [23,109].

1.5 Aims of the thesis

It is now well recognized that bacteria are very diverse, ubiquitous, social and live in complex communities, often exposed to heterogeneous fluctuating environments. Members of bacterial communities typically form networks of antagonistic, mutualistic or other interactions with each other, and can also be connected via gene flow [74,110]. Many of the things we know about bacteria come from laboratory experiments, by observing evolution and behaviour of bacteria in vitro. However, little is known whether the many principles discovered in the contrived laboratory systems also apply in nature. This thesis bridges the gap between laboratory and nature by studying environmental pseudomonads, as evolved in nature. Specifically, my aim was to understand sociality of environmental pseudomonads mediated by the siderophore pyoverdine, and environmental and genetic determinants of this sociality, pyoverdine production and non-production. I tackled this in the two research projects presented here. I first isolated 930 pseudomonads from 48 replicated soil and pond communities, and then looked at the phenotypes and social interactions among the isolates within communities. Furthermore, I measured different environmental parameters of the habitats, to relate the phenotypes and sociality of the isolates to the abiotic factors.

In the project 1, I tested whether pyoverdine non-producers are common in nature and whether they can cheat on producers or are inhibited by them. I further uncovered the genetic basis of pyoverdine non-production and the observed social interactions. I worked with 315 *Pseudomonas* isolates from eight soil and eight pond communities. I used a combination of phenotypic screens (measured their growth and pyoverdine production under iron limitation), supernatant and pyoverdine cross-feeding assays, fluorescent tagging of non-producers, and competition assays between community members.

In collaboration with Dr. Stefan Wyder (UZH) and Dr. Michael Baumgartner (UZH) I also linked pyoverdine production profiles to the phylogeny of the isolates, sequenced and analysed the genomes of 24 pyoverdine producers and non-producers. Specifically, we reconstructed their pyoverdine locus, predicted pyoverdine molecule structure and screened for the presence of pyoverdine receptors.

In the project 2, I aimed to understand the possible abiotic and biotic determinants of (i) level of pyoverdine production, (ii) loss/absence of pyoverdine locus, (iii) non-producers' ability to exploit pyoverdine and (iv) competitiveness of pyoverdine for iron (i.e. its inhibitory effect towards pyoverdine non-producers) in *Pseudomonas* communities. Particularly, I was interested how these pyoverdine-related parameters vary as a function of the environmental structuring (soil vs. pond), environmental pH, total iron and carbon concentrations, or community diversity. In this project, I worked with 930 pseudomonads, which I isolated from 24 soil and 24 pond communities. I first carried out a phenotypic screen, supernatant cross-feeding assays, and PCR analysis for the presence of a pyoverdine locus. Then, in collaboration with Dr. Jos Kramer, I used a PCA (principal component analysis) in combination with linear-mixed models, to relate environmental factors to the phenotypes (i to iv) of the isolates.

Chapter 2. Project 1

‘My dear, here we must run as fast as we can, just to stay in place.

And if you wish to go anywhere you must run twice as fast as that.’

~ Lewis Carroll, Through the Looking-Glass, and What Alice Found There (1871)

2.1 Siderophore cheating and cheating resistance shape competition for iron in soil and freshwater *Pseudomonas* communities

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Siderophore cheating and cheating resistance shape competition for iron in soil and freshwater *Pseudomonas* communities

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All social organisms experience dilemmas between cooperators performing group-beneficial actions and cheats selfishly exploiting these actions. Although bacteria have become model organisms to study social dilemmas in laboratory systems, we know little about their relevance in natural communities. Here, we show that social interactions mediated by a single shareable compound necessary for growth (the iron-scavenging pyoverdine) have important consequences for competitive dynamics in soil and pond communities of *Pseudomonas* bacteria. We find that pyoverdine non- and low-producers co-occur in many natural communities. While non-producers have genes coding for multiple pyoverdine receptors and are able to exploit compatible heterologous pyoverdines from other community members, producers differ in the pyoverdine types they secrete, offering protection against exploitation from non-producers with incompatible receptors. Our findings indicate that there is both selection for cheating and cheating resistance, which could drive antagonistic co-evolution and diversification in natural bacterial communities.

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While microbes have become model organisms to study the evolution of cooperation in laboratory settings, we still know little about the role of microbial cooperative interactions in complex natural communities. Perhaps the most common form of microbial cooperation is the secretion of so-called public goods—compounds that are costly to produce but generate benefits for other cells in the vicinity of the producer[1]. Such public goods include matrix components to build up biofilms, enzymes to digest food, biosurfactants for cooperative swarming and iron-scavenging siderophores[1]. Many laboratory studies focused on the problem of cheating, a scenario where mutants that no longer contribute to public goods undermine cooperation by capitalising on the public goods secreted by others[2–6]. This body of work has become a paradigm for the public-goods dilemma, showing how a trait that is beneficial for the group can be selected against by the spread of selfish individuals[7]. While highly influential as a general proof of social evolution theory, a key open question is whether cheating and the public-goods dilemma also occur in natural microbial communities[8–10].

Here, we tackle this question by examining the potential for public-goods cooperation and cheating among pseudomonads from natural communities both at the genetic and behavioural level. *Pseudomonas* is a diverse genus of γ-proteobacteria, occupying a wide range of habitats (e.g. soil, aquatic ecosystems and animal hosts)[11]. Albeit diverse, many fluorescent pseudomonads share an important trait: they can produce and secrete pyoverdine, a siderophore that scavenges insoluble or host-bound iron from the environment[11]. Laboratory experiments have shown that pyoverdine is a public good that can be shared among cells, and be exploited by cheating mutants[2, 12]. Pyoverdine is a secondary metabolite produced via non-ribosomal peptide synthesis. The molecule consists of a conserved chromophore (making this molecule naturally fluorescent), an acyl side chain linked to the chromophore, and a variable peptide chain (6–12 amino acids)[13, 14]. The different *Pseudomonas* strains often produce slightly different pyoverdines, varying in the length and the composition of the peptide chain[15]. Moreover, while pyoverdine and its cognate receptor are typically specific, strains can also have less specific and/or several different receptors allowing the uptake of heterologous pyoverdines[16–19]. This pyoverdine-receptor diversity could facilitate different types of social interactions among co-occurring species. For instance, pyoverdine-producing strains could exploit each other's pyoverdines[19, 20]. Alternatively, pyoverdine non-producers could gain a foothold by exploiting foreign pyoverdines (i.e. could act as cheats)[21, 22]. Moreover, some strains might produce exclusive pyoverdine types, which remain inaccessible for competing non-isogenic strains because they lack a matching receptor—a scenario that could confer resistance to cheating[17, 23].

Although it has long been conjectured that the above-mentioned interactions could be important drivers of ecological and evolutionary dynamics in microbial communities[17, 22, 24–27], there is a lack of studies that have systematically examined siderophore-mediated social interactions, and the resulting fitness consequences among natural isolates in replicated communities. To date, the most comprehensive evolutionary study on siderophore-mediated interactions used a combination of whole-genome sequencing and phenotype screening to show that many marine *Vibrio* strains have lost the siderophore-synthesis cluster, but kept the receptor for uptake[26]. While this genomic pattern is compatible with the idea of siderophore non-producers being cheats, a direct demonstration of cheating during one-to-one strain competition is missing. Here, we build on the work by Cordero et al.[26] and demonstrate that: (a) pyoverdine non-producers co-occur with producers in soil and freshwater communities of *Pseudomonas*; (b) non-producers can exploit siderophores of certain community

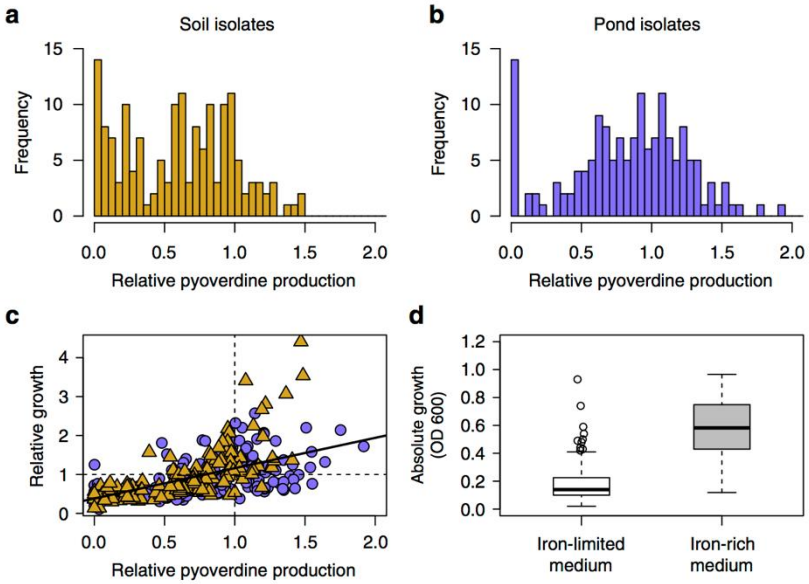

Fig. 1 Pyoverdine production and growth of natural *Pseudomonas* isolates. The natural soil (**a**, $n = 158$) and pond (**b**, $n = 157$) isolates varied greatly in their levels of pyoverdine production. Pyoverdine production was measured in iron-limited casamino acids (CAA) medium, and was scaled relative to the production levels of characterised laboratory reference strains (Supplementary Table 1). **c** There was a significant positive correlation (*solid line*) between growth of the isolates in iron-limited media and their pyoverdine production level, suggesting that pyoverdine is important for growth. Values are given as means across three replicates for soil (*yellow triangles*) and pond (*purple circles*) isolates. *Dotted lines* denote growth and pyoverdine production of reference strains. **d** While the growth of natural isolates was strongly limited in media with low iron availability, all strains grew well when iron (40 μM $FeCl_3$) was added. This demonstrates that all natural isolates could use CAA as a nutrient source. *Box plots* show the median, first and third quartile, and the 95% confidence interval

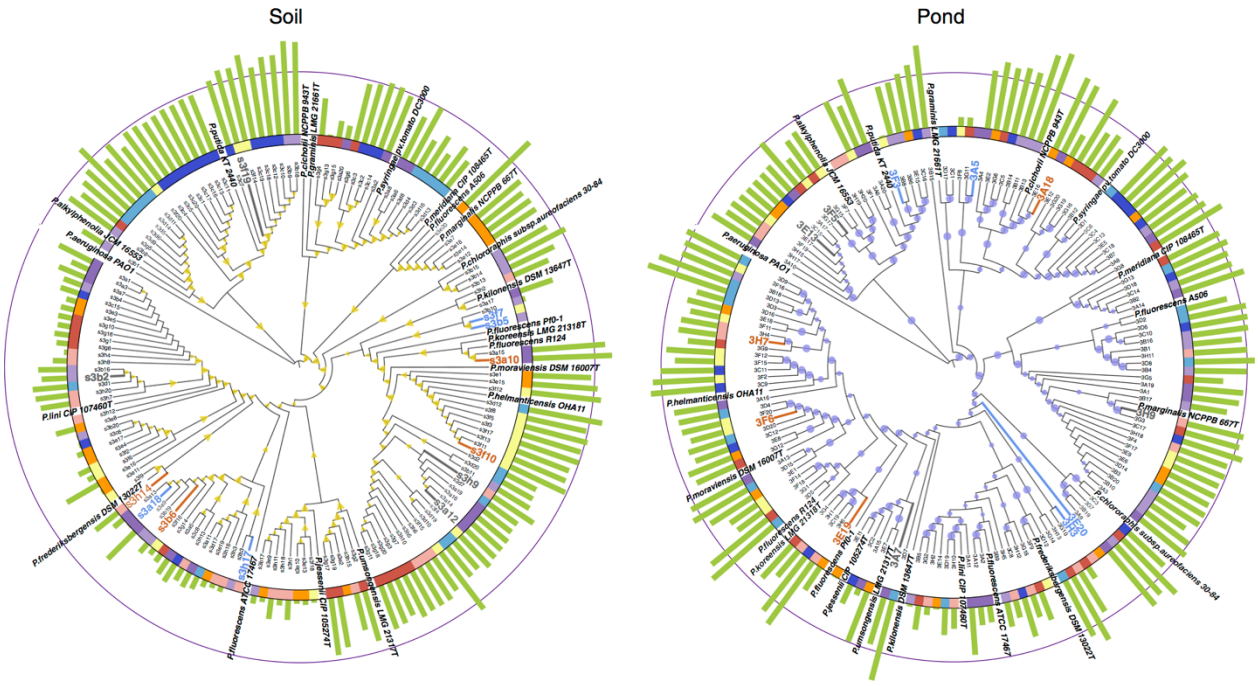

Fig. 2 Maximum-likelihood cladograms for soil and pond isolates based on partial *rpoD* sequences. For both habitats, *P. aeruginosa* PAO1 was used as the outgroup, and published *rpoD* sequences of 20 members of the *P. fluorescens* lineage (*strain names in bold*) were integrated into the cladograms to demonstrate phylogenetic affiliation and diversity of our natural isolates. *Yellow triangles* and *purple circles* indicate bootstrap values (50–100%) for branches in the soil ($n = 148$) and the pond cladogram ($n = 149$), respectively. *Green bars* depict pyoverdine production levels of isolates relative to the laboratory reference strains (*grey ring*). *Colour strips* around cladograms represent the different communities from which isolates originated. IDs for strains used for pyoverdine cross-feeding and competition assays are *enlarged*, and the different colours depict pyoverdine non-producers (*light blue font*), producers with a growth-stimulatory pyoverdine (*orange font*) and producers with a non-growth-stimulatory pyoverdine (*grey font*)

members but not of others; (c) exploitation can lead to cheating, where non-producers gain a relative fitness advantage over producers in direct competition; (d) certain pyoverdines inhibit rather than promote the growth of non-producers; and (e) the patterns of cheating and growth inhibition can be explained by receptor compatibility and pyoverdine differences between strains at the molecular level. Taken together, our findings suggest that selection for cheating and resistance to cheating could spur antagonistic co-evolution and strain diversification in natural bacterial communities.

Results

Natural strains vary in their pyoverdine production levels. We isolated 320 putative *Pseudomonas* strains from a total of eight soil and eight pond communities. For all isolates, we sequenced the *rpoD* gene (a commonly used phylogenetic marker for this genus[28]) to confirm that 315 isolates are pseudomonads. To investigate whether pyoverdine non-producers co-exist with producers in the same community, we measured the pyoverdine production profile of all 315 isolates in iron-limited casamino acids (CAA) medium. This assay revealed that strains producing no or residual amounts of pyoverdine (i.e. producing less than 5% compared to the laboratory reference strains listed in Supplementary Table 1) occurred in 14 of the 16 communities, with an overall abundance of 8.9% in both pond and soil communities. We further found that pyoverdine production is a continuous trait, with the production level ranging from zero to very high amounts (Figs. 1a, b). Variation in pyoverdine production levels was high in all communities (mean coefficient of variation, CV ± SE for soil communities: 61.8 ± 7.3%; for pond communities: 49.6 ± 3.4%; Supplementary Fig. 1a), indicating that non-, low- and high-producer strains typically co-exist.

Pyoverdine is important for growth under iron limitation. Compatible with the view that pyoverdine is important for iron acquisition in these pseudomonads, we observed a significant positive correlation between the isolates' pyoverdine production levels and their growth in CAA medium, where iron was either bound to human apo-transferrin (Fig. 1c; linear mixed model (LMM): $t_{298} = 12.0$, $p < 0.001$) or the synthetic chelator 2,2′-dipyridyl (Supplementary Fig. 2a; LMM: $t_{298} = 19.67$, $p < 0.001$). To confirm that it is indeed the amount of pyoverdine that determines growth in iron-limited CAA and not the inability of certain strains to consume the provided nutrients, we further cultured the isolates in CAA supplemented with 40 µM $FeCl_3$. This experiment revealed substantial growth for all strains, showing that all environmental isolates can use CAA as a nutrient source (Fig. 1d, paired *t*-test comparing growth of each strain in iron-limited vs. iron-rich CAA: $t_{314} = -39.40$, $p < 0.001$).

Linking pyoverdine production profiles to phylogeny. To explore the relationship between phylogeny and pyoverdine production, we constructed maximum-likelihood phylogenetic trees based on partial *rpoD* gene sequences. We constructed separate trees for soil and pond habitats, and mapped pyoverdine production profiles of strains onto the trees (Fig. 2). The absolute phylogenetic diversity was significantly higher for pond than for soil communities (median ± (1st quartile | 3rd quartile) of Faith's phylogenetic diversity for soil communities: 1.43 ± (0.97 | 1.63); for pond communities: 2.49 ± (2.22 | 2.63); Mann–Whitney *U*-test: $W = 59$, $p = 0.003$). Conversely, the CV of phylogenetic

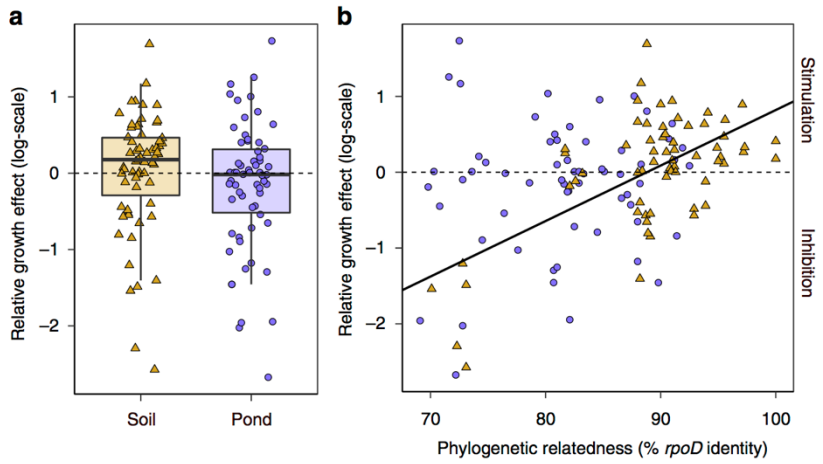

Fig. 3 Foreign supernatants containing pyoverdine can have stimulatory, neutral or inhibitory effects on the growth of receiver strains. Supernatants containing pyoverdine from donor strains were fed to receiver strains from the same community, which produced lower amounts of pyoverdine than the donors. **a** The growth effect of foreign supernatants on receivers varied on a continuum from high inhibition to high stimulation. The average growth effect of donor supernatants on receivers did not significantly differ between soil (*yellow triangles*, $n = 63$ strain combinations) and pond (*purple circles*, $n = 62$) isolates. *Box plots* depict median, first and third quartile, and the 95% confidence interval. **b** There was a significant positive correlation (*solid black line*, linear mixed model: $t_{38} = 5.32$, $p < 0.001$) between the *rpoD* identity (i.e. relatedness) of receiver–donor pairs and the supernatant growth effect for soil, but not for pond isolates. The *dashed line* depicts neutral effects, whereas values greater or smaller than zero indicate cases of stimulation or inhibition, respectively, relative to conditions where receivers grew without donor supernatant. Data were ln-transformed prior to analysis

distance within community (a relative measure of diversity) did not differ between soil and pond, and was high for both habitats (mean CV ± SE for soil: 67.9 ± 6.6%; for pond: 58.9 ± 2.6%; *t*-test: $t_{14} = 1.26$, $p = 0.227$; Supplementary Fig. 1b). These analyses show that pseudomonads generally live in diverse, multi-strain communities.

We then examined whether there is a phylogenetic signal for pyoverdine production (i.e. whether closely related strains show similar production profiles). For this analysis, we calculated Blomberg's *K* for each community, whereby, $K = 1$ indicates a phylogenetic signal as expected under the Brownian motion model of character evolution, and *K*-values close to zero stand for weak phylogenetic signals[29]. While we found a moderate phylogenetic signal for pond communities (mean ± SE across communities: $K = 0.254 \pm 0.072$), there was no phylogenetic signal for pyoverdine production in soil communities ($K = 0.009 \pm 0.005$; *t*-test for difference between habitats: $t_{14} = 3.37$, $p = 0.005$). These analyses highlight that pyoverdine production levels are not phylogenetically fixed, but highly vary even among closely related strains.

Supernatants with pyoverdine affect growth of receivers. We carried out a supernatant assay to test whether pyoverdine secreted by producers stimulates or inhibits the growth of community members producing no or little pyoverdine. From each community, we chose three strains producing no or low amounts of pyoverdine (henceforth called receivers) and fed them with the supernatant of three random strains (henceforth called donors), which produced higher amounts of pyoverdine than the receivers. Thus, each receiver was fed with pyoverdine-containing supernatants from three donors. This supernatant assay revealed that donor supernatants could both stimulate and inhibit the growth of receivers, with the effects varying on a continuum from complete inhibition to strong stimulation (Fig. 3a). The average effect of supernatants on growth did not significantly differ between soil and pond isolates (linear mixed model, LMM: $t_{14} = 0.731$, $p = 0.477$). Interestingly, the growth effect correlated positively with the phylogenetic relatedness (based on *rpoD* sequences) between receivers and donors in soil but not in pond communities (Fig. 3b; LMM for pond: $t_{37} = 0.69$, $p = 0.492$; for soil: $t_{38} = 5.32$, $p < 0.001$). This result indicates that receivers from soil communities tended to be stimulated by more closely related strains. In pond communities, on the other hand, relatedness between receiver and donor pairs was generally lower than in soil (the highest receiver–donor *rpoD* identity was 92.5%, compared to 100% among soil isolates), and receivers were often stimulated by more distantly related donors (Fig. 3b).

The supernatant assays further revealed a significant donor effect (ANOVA: $F_{47,76} = 3.90$, $p < 0.0001$) and a receiver effect approaching significance (ANOVA: $F_{43,80} = 1.49$, $p = 0.062$). This means that supernatants of donors generally had consistent (either stimulating, neutral or inhibiting) effects on multiple receivers, and that receivers were often similarly affected by foreign supernatant regardless of the identity of the donor.

Pyoverdine is responsible for the observed growth effects. We then examined whether the above-reported stimulatory and inhibitory effects are indeed triggered by pyoverdine. To test this, we first randomly picked eight non-producers (four isolates were complete non-producers, i.e. relative pyoverdine production was indistinguishable from background fluorescence; and four isolates produced residual amounts of pyoverdine, i.e. 1.4–3.1% of laboratory reference strains; Table 1) from different soil and pond communities. We then grew each non-producer under iron-limited conditions with or without a purified pyoverdine from a producer from the same community, which showed a stimulatory effect on the non-producer in the supernatant assay (Fig. 3). We found a perfect match between the two assays (Fig. 4): all the eight pyoverdines isolated from strains previously shown to be stimulatory significantly promoted the growth of the non-producers. This suggests that these eight non-producers possess receptors to exploit the supplemented heterologous pyoverdines.

In a next assay, we fed the same eight non-producers with a purified pyoverdine from producers, which showed a neutral (two cases) or inhibitory (six cases) effect in the supernatant assay.

Table 1 Characterisation of the pyoverdine locus and prediction of the pyoverdine amino acid backbone for natural Pseudomonas isolates

Community ID	Strain ID	Strain classification	Pyoverdine production (%)[a]	Number of genes						Predicted pyoverdine peptide sequence[b,c]	
				Regulator	Pyoverdine synthesis	Transport periplasma	Maturation periplasma	Secretion	Entire cluster		
				pvdS	pvdL pvdI pvdJ pvdD pvdH pvdA	pvdE	pvdQ pvdO pvdN pvdM pvdP	opmQ pvdR pvdT			
soil a	s3a18	Residual non-producer	2.8	1	5	1	5	3	15	Lys-Orn-Gly-Gly-Thr-Ser-Orn	●
	s3a10	Stimulating producer	127	1	5	1	5	3	15	Ser-Orn-Gly-Gly-Thr-Gly-X	■
	s3a12	Non-stimulating producer	64	1	6	1	5	3	16	Lys-Asp-Gly-Thr-Gly-Orn	◆
soil b	s3b5	Residual non-producer	1.4	1	6	1	5	3	16	Ser-Lys-X-Thr-Ser-Orn	□
	s3b6	Stimulating producer	20	1	5	1	5	3	15	Lys-Orn-Gly-Gly-Thr-Ser-Orn	●
	s3b2	Non-stimulating producer	42	1	7	1	5	3	17	Gly-Orn-Gly-Gly-Ser-Gly-Asp-Thr	
soil f	s3f7	Residual non-producer	2.0	1	6	1	5	3	16	Ser-Lys-X-Thr-Ser-Orn	□
	s3f10	Stimulating producer	97	1	5	1	5	3	15	Ser-Orn-Gly-Gly-Thr-Gly-X	■
	s3f19	Non-stimulating producer	120	1	7	1	5	3	17	Asp-Lys-Asp-Ser-Thr-Gly-Thr-Lys-X	
soil h	s3h17	Complete non-producer	0.02	1	-	-	1	-	2	N/A	
	s3h14	Stimulating producer	46	1	5	1	5	3	15	Gly-Lys-Thr-Ser-X-Orn	✧
	s3h9	Non-stimulating producer	77	1	6	1	5	3	16	Lys-Asp-Gly-Thr-Gly-Orn	◆
pond A	3A5	Complete non-producer	0.001	1	1	1	1	-	4	N/A	
	3A18	Stimulating producer	139	1	7	1	5	3	17	Lys-Asp-Thr-Thr-Gly-Asp-Ser	
	3A7	Non-stimulating producer	90	1	5	1	5	3	15	Gly-Lys-Thr-Ser-X-Orn-Thr-Thr	✧
pond E	3E20	Complete non-producer	0.01	1	-	-	1	3	5	N/A	
	3E19	Stimulating producer	51	1	6	1	5	3	16	Lys-Orn-Gly-Gln-Gly-Ser-Orn	
	3E13	Non-stimulating producer	61	1	7	1	5	3	17	Asp-Thr-Gly-Asp-Gln-Gln-Gly	
pond F	3F3	Residual non-producer	3.1	1	7	1	4	3	16	Asp-Lys-Orn-Thr-Gly-Ser-Ser-Orn	
	3F6	Stimulating producer	63	1	6	1	5	3	16	Lys-Asp-Gly Thr-Gly-Orn	◆
	3F5	Non-stimulating producer	48	1	7	1	5	3	17	Gly-X-X-Asp-X-Orn	
pond H	3H3	Complete non-producer	0.01	1	1	1	1	3	7	N/A	
	3H7	Stimulating producer	72	1	6	1	5	3	16	Lys-Asp-Gly-Thr-Gly-Orn	◆
	3H9	Non-stimulating producer	108	1	6	1	5	3	16	Ser-Lys-Thr-Ser-X-Orn-Thr-Thr-X	

[a]Pyoverdine production (in percentage) was measured relative to the reference strains (Supplementary Table 1)
[b]X = unknown amino acid (no significant hit with prediction software)
[c]Symbols depict pyoverdines with identical peptide backbone composition

In five cases results were consistent across the two assays: pyoverdines isolated from strains previously shown to be inhibitory significantly compromised the growth of the non-producers. Although the match was not perfect in the remaining three cases (Fig. 4, community a and b: neutral effect in the supernatant assay vs. inhibition in the pyoverdine cross-feeding assay; community A: inhibitory effect in the supernatant assay vs. neutral effect in the pyoverdine cross-feeding assay), pyoverdine never had a stimulatory effect. These findings strongly suggest that these non-producers lack receptors for the uptake of this second batch of pyoverdines. In this scenario, growth suppression can arise because incompatible pyoverdines lock away iron in the media, thereby further reducing the availability of this essential element for non-producers.

Cheating and cheating resistance in direct competition. The above findings indicate that it is mainly pyoverdine that drives the interaction patterns between our natural isolates under iron limitation. Consequently, we sought to understand how pyoverdine-mediated growth effects (ranging from stimulation to inhibition) impact the competitive abilities of strains. Accordingly, we carried out 16 direct pairwise competition assays where we mixed the eight non-producers with either their stimulating or non-stimulating (neutral or inhibitory) pyoverdine producers. To be able to distinguish the two competing strains, we integrated a constitutively expressed, fitness-neutral, mCherry marker into the chromosome of the non-producers (Supplementary Fig. 3).

When grown as monocultures, the non-producers grew significantly worse than the producers (Fig. 5a, paired *t*-test for non-producers vs. stimulating producers: $t_7 = -3.87$, $p = 0.006$; non-producers vs. inhibiting producers: $t_7 = -9.64$, $p < 0.001$). These growth patterns indicate that it is a handicap to be a pyoverdine non-producer in iron-limited medium. In contrast, fitness patterns reversed in direct competition for four strain pairs, where the non-producers could significantly outcompete their stimulating producers (Fig. 5b). This finding strongly

suggests that non-producers can act as cheats by successfully exploiting the pyoverdine secreted by producers, thereby gaining a relative fitness advantage. However, our competition assays also revealed that the ability to use a heterologous pyoverdine is not necessarily enough to gain a relative fitness advantage, as evidenced by the four cases where the non-producers lost in competition against producers secreting a compatible pyoverdine (Fig. 5b). Finally, we found that the non-producers performed worse and were strongly outcompeted in co-cultures with producers secreting an incompatible pyoverdine (Fig. 5b).

The genetic basis of pyoverdine-mediated social interactions. The results from the pyoverdine cross-feeding and competition assays suggest that: (a) the growth-stimulating and non-stimulating pseudomonads from the same community produce different pyoverdines; (b) the non-producers have receptors for heterologous pyoverdine uptake; and (c) the pyoverdine receptors of the non-producers are more similar to the receptors of the stimulating than the non-stimulating producers. To test these hypotheses, we sequenced the whole genome of the 24 strains used in the pyoverdine cross-feeding and competition assays (Figs. 4 and 5).

We first compared the organisation of the pyoverdine locus of each strain to that of previously characterised pseudomonads[14, 17, 30]. We found that each producer has a single complete pyoverdine locus, consisting of genes encoding the iron-starvation sigma factor *pvdS*, the pyoverdine-synthesis machinery (i.e. the non-ribosomal peptide synthesis assembly line), the export elements required for secretion, and a receptor (i.e. a *fpvA* homologue) for uptake (Table 1). In contrast, the four complete non-producers have a highly truncated pyoverdine cluster, where large genomic regions coding for the synthesis machinery are missing. In contrast, *pvdS* and the receptor gene are still present in these strains (Table 1). The four strains producing residual amounts of pyoverdine, meanwhile, all have a complete pyoverdine cluster (Table 1). The reason why the latter strains are unable to produce wild type amounts of pyoverdine must therefore reside in alterations of regulatory elements, as was found to be the case for evolved *P. aeruginosa* pyoverdine non-producers[31, 32]. Because the exact pyoverdine regulon is unknown for our natural isolates, it was impossible to directly test this hypothesis. In summary, our assembly analysis revealed that there are two types of non-producers: structural non-producers with a truncated pyoverdine locus and silent non-producers with a complete, yet largely inactive locus.

To investigate hypothesis (a), we used the non-ribosomal peptide synthesis assembly line identified in the producers to predict the peptide sequence of the pyoverdine backbone. These analyses indicate that the stimulating and the non-stimulating producers, from the same communities, produce structurally different pyoverdines (Table 1), which means that each non-producer was indeed confronted with two different types of pyoverdines in our assays. When focusing on pyoverdine uptake, we found that all the non-producers have multiple homologues of the *fpvA* receptor in their genome (Table 2). This finding supports hypothesis (b), as it shows that the non-producers (but also the producers) seem to be equipped for taking up heterologous pyoverdines. Given the many *fpvA* homologues, the direct testing of hypothesis (c) becomes difficult, as we do not know which homologue is actually used to take up the specific pyoverdine provided in our assays. Nonetheless, we performed two comparisons that serve as proxies for testing hypothesis (c). In the first of these, we compared the sequence similarity of FpvA encoded in the producer's pyoverdine locus to any FpvA homologues in the non-producer. In the second comparison, we analysed the similarity of the FpvA sequence encoded in the pyoverdine locus (in residual non-producers) or close to its remains (in complete non-producers) to any of the FpvA homologues in the producers. Both comparisons were in support of hypothesis (c): receptor similarities were higher between the non-producers and their corresponding stimulating producers than between the non-producers and the non-stimulating producers (comparison 1: respective FpvA similarities (mean ± SE) were 0.58 ± 0.05 vs. 0.40 ± 0.01, paired *t*-test: $t_7 = 3.61$, $p = 0.009$; comparison 2: respective FpvA similarities were 0.79 ± 0.05 vs. 0.48 ± 0.11, paired *t*-test: $t_7 = 3.15$, $p = 0.016$; Table 2).

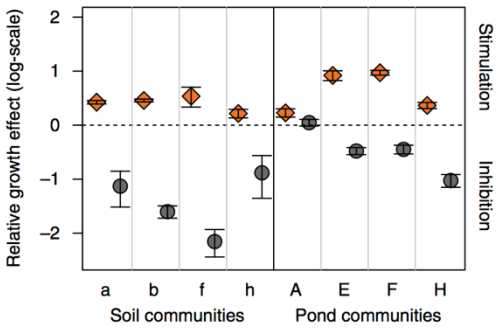

Fig. 4 Heterologous pyoverdines promote or inhibit growth of receiver strains. Eight pyoverdine non-producers, each originating from a different community, were supplemented with purified pyoverdine from either (i) a strain, which showed a stimulatory effect (*orange diamonds*) in the supernatant assay, or (ii) a strain, which showed neutral (two cases) or inhibitory (six cases) effect (*grey circles*) in the supernatant assay. The *dashed line* depicts neutral effects, whereas values greater or smaller than zero indicate cases of growth stimulation or inhibition, respectively, relative to conditions where receivers grew without heterologous pyoverdine. The data show a good match between the supernatant and the pyoverdine cross-feeding assays, suggesting that it is indeed heterologous pyoverdine that triggers the patterns of growth stimulation and inhibition reported in Fig. 3. Data points show means ± 95% confidence intervals across five replicates. Data were ln-transformed prior to analysis

Discussion

Cheating is characterised by individuals exploiting the benefits of cooperative acts performed by others[33]. This phenomenon has been extensively studied in microbial laboratory systems in the context of fruiting body[34, 35]and biofilm[6, 36] formation, group defence strategies[37, 38], swarming motility[5, 39], enzyme[3, 4], toxin[40] and siderophore[2, 12, 41–43] production. Although social interactions and cheating seem to cover all aspects of microbial life, their role in natural microbial communities remains largely unclear (apart from fruiting body formation)[44–47]. Our study tackled this gap in knowledge and shows that social interactions mediated by shareable iron-scavenging pyoverdines can have important consequences for strain–to–strain interactions in phylogenetically diverse natural soil and pond *Pseudomonas* communities. In particular, we found that: (a) strains that produce no or low amounts of pyoverdine commonly occur in natural communities, although iron is a key growth-limiting factor for pseudomonads in natural habitats[11]; (b) there are two types of non-producers, which both likely evolved from ancestral producers: structural non-producers with a truncated pyoverdine locus and silent non-producers with a complete, yet largely inactive locus; (c) the non-producers possess multiple pyoverdine receptors, and they are able to capitalise on

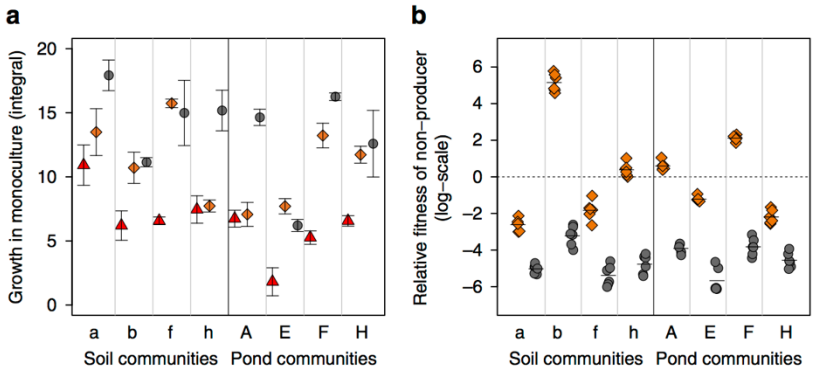

Fig. 5 Direct competition between pyoverdine producers and non-producers reveal both cheating behaviour and resistance to cheating. **a** When grown as monocultures, the eight pyoverdine non-producers grew significantly worse than the pyoverdine producers, showing that the inability to produce pyoverdine represents a handicap in iron-limited CAA medium. Growth of non-producers (*red triangles*) tagged with a neutral constitutive mCherry marker was compared to growth of the strains producing a pyoverdine type that can (*orange diamonds*) or cannot (*grey circles*) be used by the respective non-producers for iron uptake. Data points show means ± 95% confidence intervals across three replicates. **b** Direct competition between non-producers and producers revealed three different scenarios. When competing against producers that secrete a compatible pyoverdine (*orange diamonds*), non-producers could win the competition in four cases. In the other four cases, non-producers could not outcompete producers despite being able to use the producer's pyoverdine. Finally, when competing against producers that secrete an incompatible pyoverdine (*grey circles*), non-producers were always strongly outcompeted. The *dashed line* depicts fitness parity, whereas values greater or smaller than zero indicate cases where non-producers or producers won the competition, respectively. While symbols show individual data points, bars depict means across six replicates. Data were ln-transformed prior to analysis

pyoverdine produced by other community members; (d) certain non-producers can outcompete producers from the same community in direct competition through pyoverdine exploitation; and (e) some producers secrete incompatible pyoverdines that repress rather than promote the growth of non-producing community members. These results highlight that siderophores play an important, multi-faceted role in shaping social interactions between co-occurring bacterial strains, and are likely to drive diversification and competitive dynamics in natural bacterial communities.

Our study revealed that pyoverdine-mediated social interactions among natural pseudomonads are complex, and not only involve cheating, as observed for laboratory strains[2, 12, 41], but also include pyoverdine-mediated growth inhibition, and the use of heterologous pyoverdine that results in an absolute but not a relative fitness benefit for non-producers (Figs. 3–5). One reason for this increased complexity is that social interactions in our natural communities occur among phylogenetically diverse strains (Fig. 2), which differ in the pyoverdines they produce and the number and types of receptors they possess (Tables 1–2). For instance, our observation that certain non-producers can exploit and outcompete producers suggests that these non-producers possess a high-affinity receptor for specific heterologous pyoverdines. Conversely, our observation that certain non-producers are inhibited by other heterologous pyoverdines suggests that these non-producers possess incompatible receptors, which excludes them from social interactions, and thus allows producers to privatise pyoverdine and iron uptake[48]. Finally, our finding that some non-producers can exploit heterologous pyoverdines, but do not outcompete the producers, could indicate that these non-producers have receptors with relatively low affinity for these particular pyoverdines, or that non-producers were kept in check by producers through other mechanisms, such as toxin-mediated interference competition[49].

Another source of complexity is our observation that pyoverdine production is a continuous and not a binary trait, as it is typically the case in laboratory experiments where knockout mutants are used as non-producers. Genetic work on laboratory *P. aeruginosa* strains revealed that point mutations in the regulatory gene *pvdS* can lead to continuous variation in PvdS activity and levels of pyoverdine production[31, 50]. The consequence of this is that not only non-producers can exploit producers, but potentially any strain that produces a lower amount of pyoverdine than its competitor could act as a cheat[51–53]. Moreover, the continuous nature of the pyoverdine trait could favour facultative cheating[54], where strains invest to some extent in their own pyoverdine production when growing alone, but switch to the exploitation of heterologous pyoverdines when other strains are nearby. Our genomic analysis supports the idea of strains exhibiting flexible facultative strategies, as most sequenced isolates possess multiple different pyoverdine-receptor homologues (median 4, range 1–19; Table 2). Moreover, strains like those four that had a complete pyoverdine locus, yet only produced residual amounts of pyoverdine (Table 1) could be candidates pursuing facultative strategies: sustain themselves with the little amount of pyoverdine they make when growing alone, but switch to exploitation in co-culture with other producers.

We might now ask what the consequences of the reported social interactions for the long-term evolutionary dynamics in natural communities might be. Previous work proposed that one way to escape cheating is to mutate pyoverdine and receptor types[17, 25, 27]. This evolutionary response of producers could in turn impose selection on non-producers to mutate their receptors accordingly, to acquire new compatible receptors through horizontal gene transfer or to evolve broad-range receptors. These evolutionary adaptations and counter-adaptations could infinitely continue and lead to antagonistic co-evolution generating ever new variants of pyoverdines and receptors[17, 31]. Several of our findings are in line with the scenario of antagonistic co-evolution: we observed a high pyoverdine diversity among producers (i.e. the 16 producers analysed in Table 1 produced 11 different pyoverdine types). Furthermore, we found that many strains possess multiple pyoverdine receptors (Table 2), and non-producers could only use the pyoverdine of certain producers but not of others (Fig. 4). Finally, some producers were consistently resistant against exploitation by multiple non-producers, whereas other producers were particularly vulnerable to exploitation. All these findings together indicate that some strains might be ahead of the evolutionary race, by

Table 2 Numbers and similarities of the FpvA pyoverdine receptors between producers and non-producers

Community ID	Strain ID	Strain classification	Number of fpvA homologues	Highest similarity of the FpvA in the producer's pyoverdine locus to the FpvA homologues in the non-producer		Highest similarity of the FpvA in the non-producer's pyoverdine locus to the FpvA homologues in the producer
				Stimulator	Non-stimulator	Non-producer
soil a	s3a18	Residual non-producer	2	0.76	0.36	1.00
	s3a10	Stimulating producer	1	1.00	-	0.84
	s3a12	Non-stimulating producer	2	-	1.00	0.83
soil b	s3b5	Residual non-producer	4	0.44	0.38	1.00
	s3b6	Stimulating producer	2	1.00	-	0.92
	s3b2	Non-stimulating producer	2	-	1.00	0.88
soil f	s3f7	Residual non-producer	4	0.46	0.41	1.00
	s3f10	Stimulating producer	5	1.00	-	0.89
	s3f19	Non-stimulating producer	11	-	1.00	0.23
soil h	s3h17	Complete non-producer	3	0.74	0.36	1.00
	s3h14	Stimulating producer	4	1.00	-	0.89
	s3h9	Non-stimulating producer	2	-	1.00	0.83
pond A	3A5	Complete non-producer	5	0.50	0.42	1.00
	3A18	Stimulating producer	4	1.00	-	0.62
	3A7	Non-stimulating producer	4	-	1.00	0.24
pond E	3E20	Complete non-producer	7	0.45	0.37	1.00
	3E19	Stimulating producer	3	1.00	-	0.84
	3E13	Non-stimulating producer	19	-	1.00	0.24
pond F	3F3	Residual non-producer	10	0.59	0.41	1.00
	3F6	Stimulating producer	2	1.00	-	0.51
	3F5	Non-stimulating producer	8	-	1.00	0.37
pond H	3H3	Complete non-producer	9	0.69	0.47	1.00
	3H7	Stimulating producer	3	1.00	-	0.83
	3H9	Non-stimulating producer	6	-	1.00	0.25

either being particularly successful in heterologous pyoverdine exploitation or by being generally resistant to it.

For a complete understanding of the system, we would need to know how the pairwise interactions investigated in our study add up at the community level. While a definite answer is not yet possible, a recent laboratory study examined population dynamics in communities where non-producers simultaneously interacted with one producer secreting a compatible pyoverdine, and another producer secreting an incompatible pyoverdine[27]. This study revealed non-transitive competitive dynamics, where non-producers outcompeted producers with a compatible pyoverdine, but were themselves outcompeted by producers with an incompatible pyoverdine. Overall, strains chased each other in a competitive race with no overall winner, which resulted in the maintenance of biodiversity and stable community composition. Our data now reveal that cheating non-producers and cheating-resistant producers are indeed both present in our communities, which opens the possibility for these biodiversity-promoting mechanisms to operate in natural systems.

How does our work compare to the seminal study by Cordero et al.[26] who showed that siderophore non-producing and producing *Vibrio* strains co-exist with one another in a marine ecosystem? One important insight from our study is that social interactions between producers and non-producers are not restricted to marine communities, but also occur in soil and freshwater ecosystems, in a completely different taxon. This highlights that siderophore-mediated interactions between taxonomically diverse strains are likely a common feature of microbial communities. Another similarity between the two studies is that there is no strong phylogenetic signal for siderophore production (Fig. 2). This means that pyoverdine non- or low producers were not limited to a few specific taxonomic clades, but occurred across the entire phylogenetic tree. This indicates that, as for the *Vibrio* system, non-producers frequently arise de novo from within producer clades. However, the mechanism by which non-producers evolve differs between the *Vibrio* and our *Pseudomonas* system. Particularly, Cordero et al.[26] found discrete phenotypes: strains were either full producers (40%) or non-producers (60%), and these phenotypes correlated well with the presence or absence of the siderophore-synthesis clusters in the genome of these strains. Conversely, in our system non-producers are relatively rare (9%) and come in two different forms: structural non-producers with a truncated pyoverdine locus and silent non-producers with a complete, yet largely inactive locus, producing only residual amounts of pyoverdine. In addition, isolates showed continuous phenotypes, from pyoverdine non- to full production (Fig. 1). This demonstrates that most isolates have not lost their pyoverdine-synthesis cluster, and suggests that modifications in regulatory elements might rather be the key determinants of how much pyoverdine a strain is capable to produce.

Another key difference between the two study systems is that structural diversity exists for pyoverdine (Table 1), whereas the

Vibrio siderophores (aerobactin and vibrioferrin) come in a single molecular form[55]. Because pyoverdine diversity could select for receptor diversity[17, 18], successful cheating is then not so much about having a receptor per se, but rather about having a matching receptor (Table 2). We can think of two scenarios of how matching receptors can be acquired. First, if non-producers evolve de novo from producers, then they inherently possess the matching receptor of the producer they originated from. This route to exploitation might commonly apply in spatially structured habitats where de novo non-producers can rely on closely related producers staying in close vicinity. Indeed, our supernatant assays suggest that pyoverdine-mediated growth stimulation preferentially occurs among closely related strains in soil, a highly structured environment (Fig. 3b). Second, non-producers could acquire matching receptors through horizontal gene transfer. This route to exploitation might preferentially occur in habitats with low spatial structure, where strains readily mix and closely related producers are not necessarily nearby. This scenario indeed seems to apply to our pond communities, living in a fairly unstructured habitat, where pyoverdine-mediated growth stimulation primarily occurred among more distantly related strains (Fig. 3b).

In conclusion, our findings demonstrate that pyoverdine-mediated cheating and competition for iron are prevalent among natural *Pseudomonas* isolates and have important fitness consequences. Because iron scavenging via siderophores is ubiquitous among bacterial taxa in iron-limited habitats[11, 55], we propose that siderophore-mediated social interactions are important in many ecosystems, and are likely involved in shaping strain diversity and community dynamics. We further propose that other microbial social traits might play similar roles. For instance, many bacterial species secrete small signalling molecules (e.g. acyl homoserine lactones, AHLs) for communication and the coordination of group-level activities[56]. It has previously been shown that AHLs are exploitable public goods[3, 4], are structurally diverse and occur in many different species[57]. The complex social interactions uncovered here for siderophores might thus well apply to AHL-based communication systems. Taken together, our study highlights that not only abiotic but also social components need to be considered in order to fully understand microbial community assembly and functioning.

Methods

Sampling and isolation of pseudomonads. We sampled 16 *Pseudomonas* communities from soil and pond habitats ($n = 8$ each) located on the campus of the University of Zurich Irchel (47.40° N, 8.54° E), Switzerland. We used the following sampling and isolation protocol, adapted from previous studies[58–60]. For soil sampling, we used a metal soil probe with a 7 mm diameter slot to sample the upper 10 cm of the soil. From the extracted soil cores, we discarded the upper and the lower 2 cm, and processed the middle part for strain isolation. For pond sampling, we collected water close to the shore from the upper layer by totally immersing 250 ml sterile glass bottles into the water. All samples were transported on ice and processed in the laboratory within 25 h. For the soil samples, approximately 1 g was suspended in 9 ml of 0.85% NaCl solution, and then vortexed vigorously for 2 min. We then plated 400 µl of 10^{-1}, 10^{-2} dilutions (in 0.85% NaCl solution) of the bacteria suspensions on agar plates containing Gould's S1 medium supplemented with 100 µg/ml of the antifungal cycloheximide and 50 µM $FeCl_3$ (to also allow potential siderophore non-producers to grow). This medium is selective for fluorescent *Pseudomonas*[61]. The pond samples were filtered through 0.22 µm PES bottle-top vacuum filter (Millipore steritop-GP 250 ml). A stirring rod and 2.5 ml 0.85% NaCl solution were added to the filter, sealed at the bottom with parafilm, and the filter with suspension was stirred for 3 min. We plated 240 µl of 0, 10^{-1} dilutions of the bacteria suspensions on Gould's S1 medium. Plates were incubated for three days at room temperature in the dark. After incubation, we picked 20 random isolates per community and streaked them out on lysogeny broth (LB) agar plates to finally isolate a single purified colony (320 in total). These isolates were grown statically in 1 ml LB for 24–48 h in 24-well plates. Glycerol was added to a final concentration of 25% to prepare freezer stocks for storage at −80 °C. Each isolate obtained an identification code, consisting of location ID ('s3': soil Irchel campus; '3': pond Irchel campus), followed by a letter (for soil communities: small letters 'a' to 'h'; for pond communities: capital letters 'A' to 'H') and a number (1–20).

rpoD amplification and sequencing. To verify that the isolates are indeed pseudomonads, we PCR amplified and sequenced a part of the housekeeping *rpoD* gene for 315 isolates (PCR or sequencing failed for 5 isolates, which were excluded from further experiments). *rpoD* is commonly used for phylogenetic affiliation of pseudomonads[28, 62]. PCR mixtures contained 2 µl of 10 µM solutions of each primer, PsEG30F and PsEG790R[28], 25 µl Quick-Load Taq 2X Master Mix (New England Biolabs), and 21 µl of sterile Milli-Q water. We added bacterial biomass either from single colonies on LB plates or directly from glycerol stocks (note that the latter method worked much better than the former one) to the PCR mixture distributed in 96-well PCR plates. Plates were sealed with an adhesive film. The following PCR conditions were used: denaturation at 94.5 °C for 10 min; 30 cycles of amplification (1 min denaturation at 94 °C, 1 min primer annealing at 55 °C, and 1 min primer extension at 68 °C); final elongation at 68 °C for 10 min (adapted from ref. [28]). The PCR products were purified and commercially sequenced using the PsEG30F and/or the PsEG790R primer.

Taxonomic and phylogenetic affiliation. We used NCBI BLASTN analysis for taxonomic assignment of *rpoD* sequences. To cover the broad species diversity of the isolates, we chose 20 characterised *Pseudomonas* all belonging to the *P. fluorescens* lineage as reference strains for phylogenetic trees (Supplementary Table 2): four main groups (*P. fluorescens*, *P. lutea*, *P. putida* and *P. syringae*), and seven subgroups of the *P. fluorescens* group (*P. chlororaphis*, *P. corrugata*, *P. fluorescens*, *P. gessardi*, *P. jessenii*, *P. koreensis* and *P. mandelii*)[63, 64]. *P. aeruginosa* PAO1 was used as an outgroup.

We retrieved partial or full sequences of all reference and outgroup strains from GenBank. We used MEGA 7 software (Supplementary Table 3) to manipulate *rpoD* sequences and construct phylogenetic trees. *rpoD* sequences of all strains were converted to amino acid sequences, and aligned with MUSCLE. We checked quality of alignments manually and removed the shortest sequences (to guarantee a sequence length ≥ 600 bp), leaving us with 297 *rpoD* sequences of natural isolates. Sequences in the alignment were trimmed at both ends to obtain maximum overlap and reconverted to nucleotide sequences, resulting in 609 bp sequences for all strains. We constructed maximum-likelihood (ML) trees, using General time reversible (GTR) + G + I model, which yielded the best fit to our data set. Bootstrapping was carried out with 100 replicates, keeping gaps. We displayed and manipulated ML trees using the iTOL web tool (Supplementary Table 3). For some analyses, we calculated the relatedness between strains by carrying out pairwise alignments of *rpoD* sequences using EMBOSS Water (Supplementary Table 3).

Measurement of growth and pyoverdine production levels. To evaluate whether natural isolates can produce pyoverdine, we grew all isolates under iron-limited conditions and assessed their pyoverdine production levels. We first grew isolates in 150 µl LB in 96-well plates overnight (16–18 h) under static conditions at room temperature. We then transferred 2 µl of overnight cultures to 200 µl iron-limited CAA medium (containing 5 g casamino acids, 1.18 g $K_2HPO_4{\cdot}3H_2O$, 0.25 g $MgSO_4{\cdot}7H_2O$ per litre) supplemented with 25 mM HEPES buffer, 20 mM $NaHCO_3$ and 100 µg/ml human apo-transferrin (a strong natural iron chelator) in a 96-well plate. All chemicals were purchased from Sigma-Aldrich, Switzerland. Each plate contained isolates from one community in triplicates and eight reference strains from our strain collection known to produce pyoverdine (Supplementary Table 1). Following 18 h of incubation at room temperature in the dark, we measured growth (optical density OD at 600 nm) and pyoverdine production levels (relative fluorescence units (RFU) with excitation: 400 nm and emission: 460 nm) with an Infinite M200 Pro microplate reader (Tecan Group Ltd., Switzerland)[65]. We then calculated the relative growth and pyoverdine production for each isolate by dividing its OD_{600} and RFU by the average respective OD_{600} and RFU of the reference strains.

We carried out control experiments to verify whether it is indeed iron limitation that induces the observed growth and pyoverdine production patterns. First, we checked whether all isolates were able to use CAA as a nutrient source by growing strains in the same way as described above, just this time with 40 µM $FeCl_3$ supplemented to CAA (Fig. 1d). Second, to rule out specific effects in response to transferrin as iron chelator we repeated the growth experiments with a synthetic iron chelator 2,2′-dipyridyl (400 µM, 24 h growth assay). These experiments yielded qualitative similar results for both iron chelators (Supplementary Fig. 2). Finally, we verified that end point OD_{600} is a reliable measure of growth by correlating this measurement to growth parameters extracted from time-course data, and by relating end point OD_{600} to actual cell counts obtained from flow cytometry (Supplementary Fig. 4). Data on growth trajectories were obtained by growing a subset of isolates ($n = 155$) in 200 µl CAA with 400 µM 2,2′-dipyridy in 96-well plates in a Tecan microplate reader at room temperature for 24 h. OD_{600} was measured every 30 min prior a 15-second shaking event (3 mm orbital displacement). Some of the natural isolates exhibited non-standard growth trajectories, characterised by a long linear rather than an exponential growth phase. Because such patterns prevent the fitting of parametric growth models, we applied

non-parametric spline fits using the R 'grofit' package (Supplementary Table 3). From these spline fits, we extracted the integral (area under the curve) as the growth parameter of interest. The integral is a representative growth measure as it combines information on the lag, growth and stationary phase in one single estimate[66]. Flow cytometry was carried out on a subset of soil ($n = 24$) and pond ($n = 24$) isolates grown in CAA medium with 200 µM 2,2-dipyridyl in a 96-well plate for 24 h. OD_{600} was measured in a Tecan microplate reader and samples were subsequently diluted 100× in 0.85% NaCl solution. Cells were fixed with glutaraldehyde (final concentration 2.5%, Sigma-Aldrich, Switzerland) and stained with Sybr Green I (5×10^{-5}dilution of commercial stock, Invitrogen, USA) for 10 min at room temperature in the dark. Samples were analysed using an InFlux V-GS cell sorter (Becton Dickinson Inc., USA). A blue laser (200 mW, 488 nm) was used for detection of side-scattered (SSC) light and Sybr Green I fluorescence (531 nm). Analysis of flow cytometry data was carried out with an in-house custom software (J. Villiger and J. Pernthaler, University of Zurich, unpublished) and bacterial cells were determined using SSC vs. green fluorescence. These control experiments revealed highly significant correlations between end point OD_{600}, growth integrals and cell counts demonstrating that using end point OD_{600} is a reliable measure of growth for the natural isolates (Supplementary Fig. 4).

Supernatant assay. For each community, we harvested supernatants from three pyoverdine-producing isolates (donors) and fed them to three receiver strains. Receivers were always the three isolates with the lowest pyoverdine production levels in the community (mean ± SE relative pyoverdine production levels of receivers: 0.183 ± 0.038, $n = 44$). In all but one case (pond community G) all three receivers differed in their *rpoD* sequence, and thus represent phylogenetically different strains. For donors, we picked three random isolates, which had higher pyoverdine production levels than the receivers (mean ± SE relative pyoverdine production levels of donors: 0.785 ± 0.056, $n = 48$).

To generate pyoverdine-containing supernatants, we grew isolates in 4 ml CAA medium with 200 µM 2,2′-dipyridyl in 14 ml polypropylene round-bottom tubes, shaken (160 rpm) at 28 °C. Supernatants were isolated in late exponential phase ($OD_{600} = 0.3–0.5$; measured with Tecan microplate reader), and centrifuged for 2 min at 7,500 rcf (Eppendorf Centrifuge 5804R). We then filter-sterilised supernatants by passing them through a 0.22 µm PES membrane filter and kept them at −20 °C. Meanwhile, we grew the receivers in 1 ml LB in 24-well plates for 24 h static at room temperature. Then, 1.5 µl of receiver cultures were added to CAA with 200 µM 2,2′-dipyridyl without or with 20 µl of donor supernatant (total culturing volume was 200 µl for both conditions) in 96-well plates in threefold replication and grown for 24 h at room temperature in a microplate reader. Growth trajectories were measured and analysed as described above. For each donor-receiver pair, we calculated the relative growth effect as the ratio of growth integrals of receivers with vs. without donor supernatant, and log-transformed this value to obtain normally distributed residuals. Values above or below zero indicate whether receivers were respectively stimulated or inhibited by the donor supernatant.

Pyoverdine cross-feeding assay. To test whether it is pyoverdine that triggers the above-observed growth effects, we performed cross-feeding assays using eight strain triplets (Table 1). We chose eight receivers, which produced less than 5% of pyoverdine compared to our reference strains. Each receiver originated from a different community (four soil and four pond communities). For each receiver, we picked two pyoverdine producers, which were previously shown to either stimulate or inhibit the growth of receivers in the supernatant assay. For one receiver, there was no inhibitor, and we thus chose a pyoverdine producer, supernatant of which had a neutral effect. We adapted the method of Meyer et al.[67] to crudely purify pyoverdine from the 16 producers (Supplementary Methods). For the cross-feeding assay, we suspended 3 mg of each purified pyoverdine in 500 µl Milli-Q water and passed the solution through a 0.22 µm PES filter. A 4 µl aliquot of the sterile pyoverdine solution was then added to 196 µl of iron-limited CAA (with 200 µM 2,2′-dipyridyl) in a 96-well plate, and inoculated with 1.5 µl of receiver LB overnight cultures in fivefold replication. As a control, we grew receivers without pyoverdine supplementation. Plates were kept for 24 h in a microplate reader. Growth trajectories were measured and analysed as described above.

Competition assays. We carried out pairwise competition assays between non-producers and their stimulatory or non-stimulatory pyoverdine producers under iron-limited conditions. To be able to distinguish the two competing strains, we used the mini-Tn7 system[68] to integrate a constitutively expressed mCherry marker into the chromosome of non-producers. Electroporation and conjugation protocols were adapted from Choi and Schweizer[68] (Supplementary Methods and Supplementary Table 4). To obtain growth profiles of the 24 strains (Fig. 5a) in iron-limited medium, we first grew mCherry-tagged non-producers and untagged producers in LB overnight. Then we adjusted cultures to $OD_{600} = 1$ with LB and added 2 µl of the adjusted cultures to 200 µl of CAA with 200 µM 2,2′-dipyridyl in 96-well plates, in triplicates. OD_{600} was measured every 30 min at room temperature (25–28 °C) for 48 h in a Tecan microplate reader. Growth curves were analysed as described above (in the section 'Measurement of growth and pyoverdine production levels'). Prior to competition, we adjusted overnight LB cultures of all strains to $OD_{600} = 1$ in LB, and mixed strains in appropriate volumetric ratios to obtain a roughly 1:1 starting frequencies (effective starting frequencies of non-producers varied between 0.27 and 0.50). A 2 µl aliquot of the strain mix was added to 200 µl iron-limited CAA (with 200 µM 2,2′-dipyridyl) in 96-well plates, where strains competed for 48 h (static incubation 25 C°) in six replicates. Following competition, the cultures were appropriately diluted in 0.85% NaCl, plated on LB-iron plates, and incubated at room temperature for 72 h. After 24–48 h, we counted total CFUs, and after 72 h, we used fluorescence imaging (Infinity3 camera system, Lumenera Corporation) to distinguish mCherry-tagged from untagged colonies using ImageJ (Supplementary Table 3). We calculated the relative fitness of the non-producer as $w_r = (a_t \times (1-a_0))/(a_0 \times (1-a_t))$, where a_0 and a_t are the initial and final frequency of the non-producer, respectively[69]. Subsequently, we log-transformed w_r, such that values of $\ln(w_r) > 0$ or $\ln(w_r) < 0$ indicate whether non-producers have won or lost the competition, respectively.

Genomic DNA isolation for whole-genome sequencing. The 24 isolates were first grown on LB agar plates and then streaked out on Gould's S1 agar medium (supplemented with 40 µM $FeCl_3$) and grown for two days at 28 °C. Single colonies were inoculated into 4 ml LB in 14 ml polypropylene round-bottom tubes and grown overnight at 30 °C shaken (200 rpm). Genomic DNA was extracted from 1 ml of cultures using GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Switzerland) according to the manufacturer's instructions, without RNase treatment. The main changes: we used a separately purchased proteinase K (20 mg/ml; Fermentas), and eluted DNA with EB elution buffer (Qiagen).

Whole-genome sequencing and bioinformatic analysis. Library preparation and sequencing of the 24 isolates was done at the Lausanne Genomic Technologies Facility with the Illumina Hiseq 2500 platform (paired end 2 × 250 bp) in Rapid Run mode. Libraries were constructed using the Truseq Nano DNA kit. Reads were filtered with Trimmomatic (Supplementary Table 3) and assembled with SPAdes 3.10.1 (Supplementary Table 3) using default parameters. Identification of putative coding sequences and their annotation were performed by the RAST automated annotation pipeline (Supplementary Table 3). The pyoverdine gene cluster was identified manually by a combination of keyword searches in the annotation and BLASTP searches with genes from the reference database against the genomes of isolates. In order to get gene-family profiles independent of annotation we also inferred an orthology using OrthoFinder (Supplementary Table 3). To compare the FpvA receptor encoded in the pyoverdine locus of the producers to receptors of the non-producers, the amino acid sequences of the FpvA receptor from the pyoverdine locus of the stimulators, and likewise the non-stimulators, were blasted against the genomes of the residual or complete non-producers. In the same way, the FpvA receptor sequence of residual and complete non-producers was compared to stimulating and non-stimulating producers. The best hits of the BLAST searches were then compared. The pyoverdine peptide structure was predicted from the amino acid sequence of non-ribosomal peptide synthetases by using PKS/NRPS analysis website (Supplementary Table 3).

Statistical analysis. We used linear and LMM models for statistical data analysis. All statistical tests are two-tailed, and p-values ≤ 0.05 were regarded as significant. Since strains isolated from the same community might not be independent from one another we built community identity as a random factor into our models. For our analysis on donor and receiver effects, we further added the strain ID of the donor as a random factor to our models. Whenever appropriate, we log-transformed (natural logarithm) data to meet the assumption of normally distributed residuals. For phylogenetic analysis we used the R package APE v3.2 (Supplementary Table 3). Blomberg's K-values were calculated with the phylosignal function from the R package picante v1.6-2 (Supplementary Table 3). All statistical analyses were carried out using R 3.1.2 program (www.r-project.org).

Data availability. The experimental and sequencing data that support the findings of this study have been deposited in the figshare repository (doi:10.6084/m9.figshare.5125093) and in the European Nucleotide Archive under the study accession number PRJEB21289 (http://www.ebi.ac.uk/ena), respectively.

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Author contributions

E.B. and R.K. designed the study, E.B. isolated the strains and performed the experiments, M.B. and S.W. did the bioinformatic analyses, and all authors analysed the data and wrote the paper.

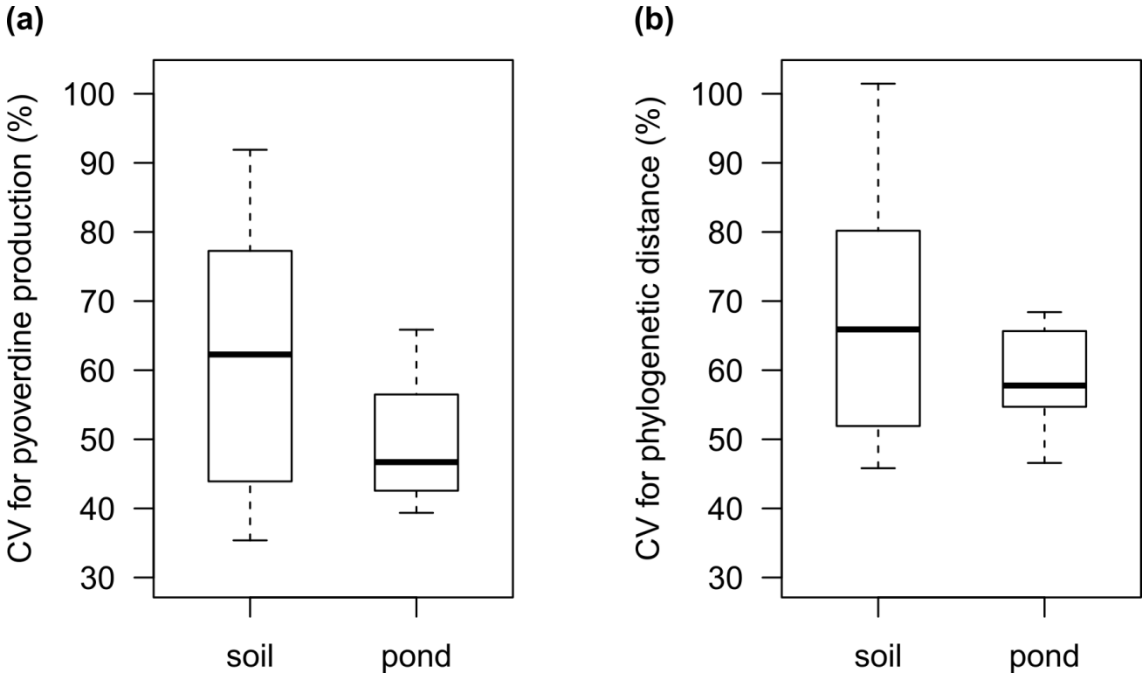
Additional information

Supplementary Information accompanies this paper at doi:10.1038/s41467-017-00509-4.

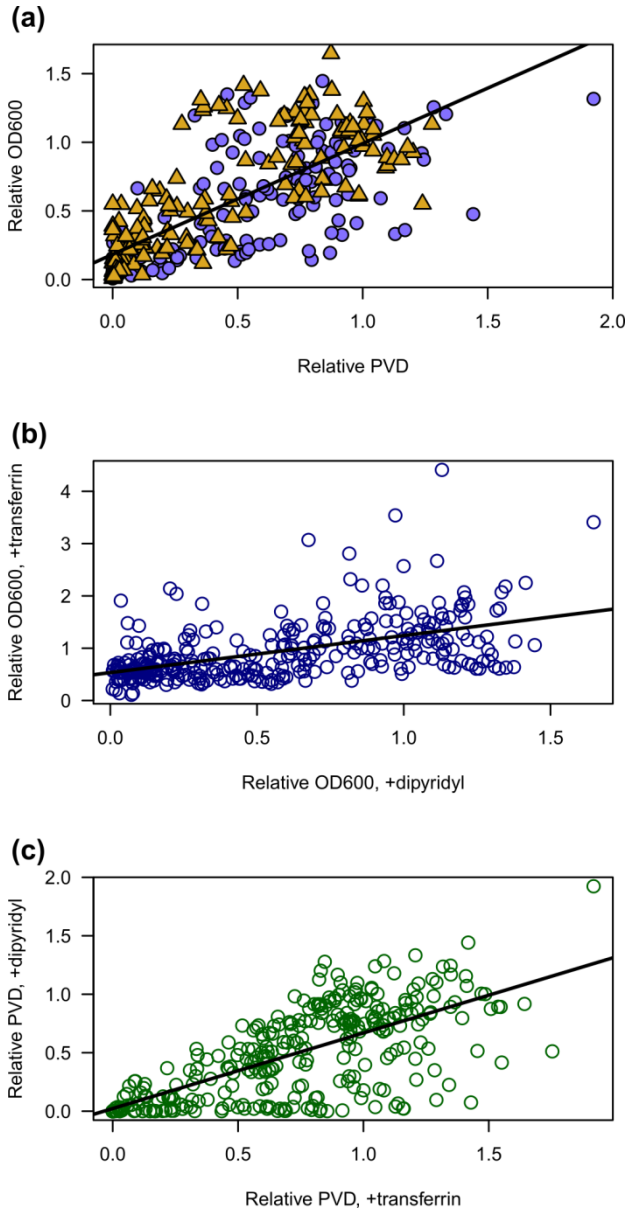
Competing interests: The authors declare no competing financial interests.

2.2 Supporting material

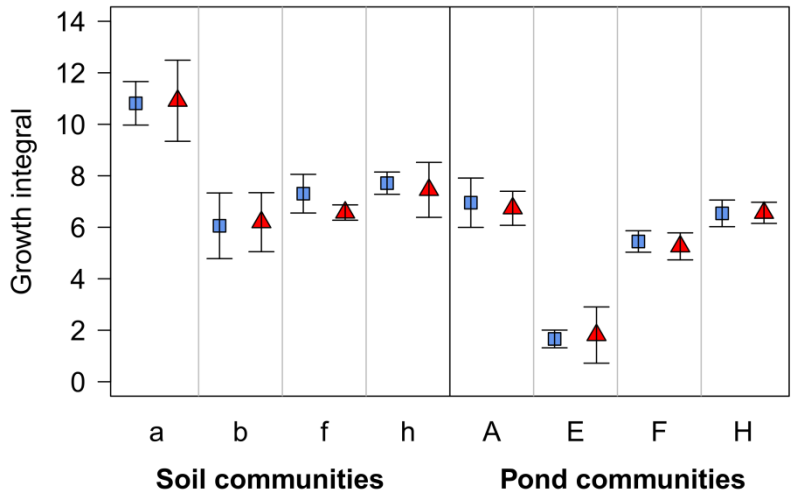
Supplementary Figures



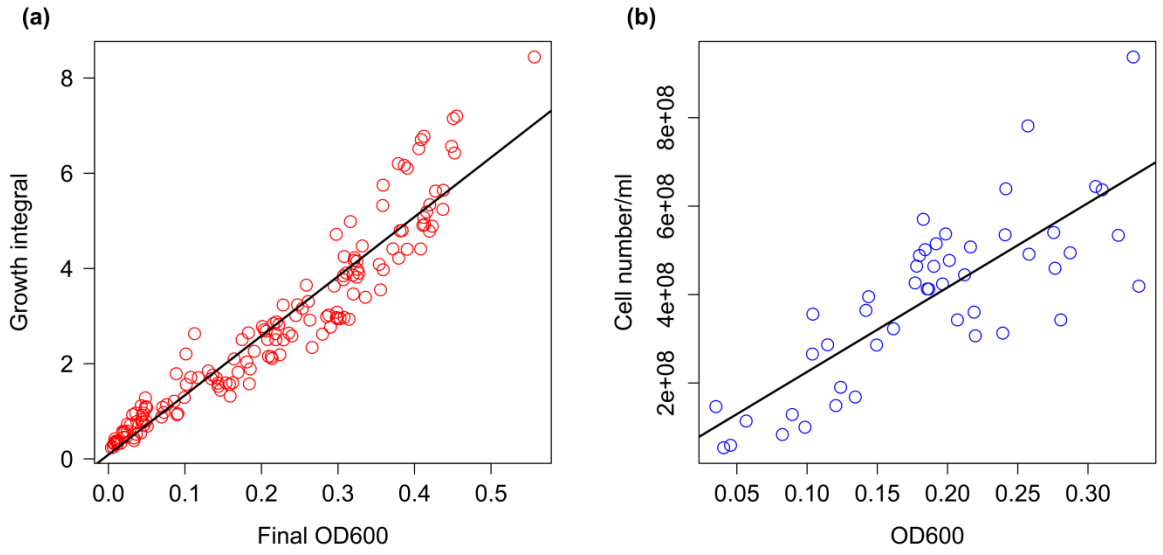
Supplementary Figure 1 | Pyoverdine production and phylogenetic diversity was highly variable in both soil and pond communities. (a) The coefficients of variation (CV = standard deviation / mean) for relative pyoverdine production was high in all communities. **(b)** The CV for phylogenetic distance was also high in both soil and pond communities. For both analyses, the CVs were calculated separately for each of the 16 communities, and based on a total of 158 soil and 157 pond isolates for (a), and 148 soil and 149 pond isolates for (b). Box plots show the median, the 1st and the 3rd quartile, and the 95% confidence interval.



Supplementary Figure 2 | Growth and pyoverdine production of natural isolates significantly correlate between different iron-limited environments. (a) Significant positive correlation between the relative growth (OD_{600}) and the relative pyoverdine production (PVD) of natural soil (yellow triangles, n = 158) and pond (purple circles, n = 157) isolates grown in CAA medium supplemented with the iron chelator 2,2'-dipyridyl (400 µM) (linear mixed model: $t_{298} = 19.67$, $p < 0.001$, solid line). **(b)** Relative growth (OD_{600}) and **(c)** relative pyoverdine production levels for isolates positively correlated between two different iron-limited media (indicated by solid lines): CAA medium with the natural iron chelator apo-transferrin versus CAA medium with the synthetic chelator, 2,2'-dipyridyl (linear models for relative OD_{600}: $t_{313} = 10.6$, $p < 0.001$, $R^2 = 0.262$; relative pyoverdine production: $t_{313} = 16.9$, $p < 0.001$, $R^2 = 0.475$). Values represent means across three replicates.



Supplementary Figure 3 | The introduction of a constitutive mCherry marker did not affect strain growth. We fluorescently tagged eight non-producers to be used in direct competition assays against producers. To test whether the fluorescent marker itself has a fitness effect, we grew tagged (red triangles) and untagged (blue squares) non-producers in iron-limited CAA medium as monocultures. There was no significant growth difference between tagged and untagged strains (paired *t*-test: $t_7 = -1.17$, $p = 0.279$). Strains were grown for 48 h as static cultures. Values are given as means ± 95% confidence intervals across three replicates.



Supplementary Figure 4 | Final OD_{600} is an accurate measure of bacterial growth and cell density. In our high-throughput assays, we used final OD_{600} as a proxy for culture growth. Because we worked with environmental isolates that differ in many aspects, we carried out two control experiments to confirm that final OD_{600} is a reliable measure of growth. **(a)** For a subset of isolates (n = 78 for soil, n = 77 for pond), we compared final OD_{600} to growth integrals obtained from 24-h kinetic growth measurements in iron-limited medium (CAA with 400 µM 2,2'-dipyridyl). We found a strong significant positive correlation between the two growth measurements (linear model: t_{153} = 45.89, $p < 0.001$, R^2 = 0.932, solid line). Values represent means across three replicates. **(b)** For another subset of isolates (each n = 24 for soil and pond), we compared final OD_{600} to cell count measures (cells/ml) obtained from flow cytometry. We also found a strong positive correlation between the two measurements of growth (linear model: t_{46} = 9.06, $p < 0.001$, R^2 = 0.633, solid line).

Supplementary Tables

Supplementary Table 1 | Reference strains used in growth and fluorescence measurement assays.

Strain	Description	Source or reference
P. aureofaciens ATCC13985	wildtype	L. Eberl strain collection, University of Zurich
P. entomophila	wildtype	L. Eberl strain collection, University of Zurich
P. protegens CHA0	wildtype	[1]
P. putida IsoF	wildtype, isolated from tomato rhizosphere	[2]
P. syringae B728a	wildtype	L. Eberl strain collection, University of Zurich
P. aeruginosa PAO1 (ATCC15692)	wildtype; pyoverdine type I	[3,4]
P. aeruginosa 2-164	CF isolate United States; pyoverdine type II	[5,6]
P. aeruginosa ATCC 013	laboratory isolate United States; pyoverdine type III	[5,6]

Supplementary Table 2 | GenBank accession or locus tag numbers of partial or complete *rpoD* sequences used as an outgroup or references for phylogenetic trees.

Strain	***rpoD* accession or locus tag**	**Group**	**Subgroup**
P. aeruginosa PAO1	NP_249267	*P. aeruginosa*	
P. chlororaphis subsp. aureofaciens 30-84	PCHL3084_RS27440	*P. fluorescens*	*P. chlororaphis*
P. kilonensis 520-20T (DSM 13647T)	AM084336	*P. fluorescens*	*P. corrugata*
P. fluorescens A506	CP003041	*P. fluorescens*	*P. fluorescens*
P. marginalis NCPPB 667	AB039575	*P. fluorescens*	*P. fluorescens*
P. meridiana CIP 108465T	FN554485	*P. fluorescens*	*P. gessardi*
P. jessenii CIP 105274T	FN554473	*P. fluorescens*	*P. jessenii*
P. umsongensis LMG 21317T	FN554516	*P. fluorescens*	*P. jessenii*
P. koreensis LMG 21318T	FN554476	*P. fluorescens*	*P. koreensis*
P. fluorescens R124	I1A_004757	*P. fluorescens*	*P. koreensis*
P. moraviensis DSM 16007T	FN554490	*P. fluorescens*	*P. koreensis*
P. helmanticensis OHA11	HG940517	*P. fluorescens*	*P. koreensis*
P. lini CIP 107460T	FN554478	*P. fluorescens*	*P. mandelii*
P. frederiksbergensis DSM 13022T	AM084335	*P. fluorescens*	*P. mandelii*
P. fluorescens ATCC 17467	AB039530	*P. fluorescens*	
P. graminis LMG 21661T	FN554469	*P. lutea*	
P. putida KT2440	NC_002947	*P. putida*	
P. alkylphenolica JCM 16553T	HE577794	*P. putida*	
P. japonica JCM 21532T	HE577795.	*P. putida*	
P. cichorii NCPPB 943	AB039526	*P. syringae*	
P. syringae pv. tomato DC3000	PSPTO_0537	*P. syringae*	

Supplementary Table 3 | Software packages used for data analysis.

Package	Application	Reference
MEGA 7 software	Phylogenetic analysis	[7]
iTOL web tool	Plotting of phylogenetic trees	[8]
EMBOSS water	Relatedness analysis between pairs of sequences	www.ebi.ac.uk/tools/psa/emboss_water/
grofit	Growth curve analysis in R	[9]
Image J	Image analysis	[10]
Trimmomatic	Filtering of sequence reads	[11]
SPAdes 3.10.1	Assembling sequence reads	[12]
RAST automated annotation pipeline	Identification and annotation of putative coding sequences	[13,14]
OrthoFinder	Identification of gene family profiles	[15]
PKS/NRPS analysis website	Predicting amino acid sequence of non-ribosomal peptide synthetases	[16]
APE v3.2	Phylogenetic analysis in R	[17]
picante v1.6-2	Calculating Blomberg's K-values	[18]

Supplementary Table 4 | Strains used for fluorescent tagging.

Strain	**Relevant properties**	**Source**
E. coli S17-1 λpir pUX-BF13	with conjugation elements and helper plasmid	L. Eberl strain collection, University of Zurich
E. coli S17 λpir miniTn7-Ptac-mCherry	with mini-Tn7 plasmid carrying mCherry under constitutive promoter	J. van der Meer, University of Lausanne
E. coli S17-1 λpir miniTn7-Ptac-mCherry	with conjugation elements and mini-Tn7 plasmid carrying mCherry under constitutive promoter	L. Eberl strain collection, University of Zurich

Supplementary Methods

Pyoverdine purification assay. We adapted the method of Meyer et al. [19] to crudely purify pyoverdine from the 16 producer strains. Briefly, we added 2 ml of producer overnight LB culture to 500 ml CAA with 200 µM 2,2'-dipyridyl in a 1 L glass flask, and let cultures grow for 24 h at 25°C shaken (100 rpm). Afterwards, we centrifuged cultures at 7,500 rcf for 15 min (in 50 ml aliquots). We acidified supernatants with HCl 1 M till pH = 6 and centrifuged again at 5,000 rcf for 10 min. The supernatant was then added on a XAD-4 (Amberlite) column with a flux of two drops per second. The column was washed with 300 ml of Milli-Q water. 50% methanol (in Milli-Q water) was subsequently used as an eluent. We collected fractions of the eluate that showed peak pyoverdine fluorescence (150 – 250 ml). The fractions were distributed in Petri dishes and left for 24 h under a hood to let methanol evaporate. The residues were first dissolved in Milli-Q water, then combined and lyophilised for 48 h (Lyovac). Columns were regenerated by washing with 1 L of methanol containing 1% of concentrated HCl (32%) and then washed with 1 L of Milli-Q water.

Fluorescent tagging. We tagged the eight non-producers with a red fluorescent mCherry protein gene via electroporation or conjugation using a mini-Tn7 system for chromosomal integration [20]. Electroporation protocol was adapted from Choi & Schweizer [21]. We used donor strains *Escherichia coli* S17 λpir and S17-1 λpir carrying a plasmid with the mini Tn7-mCherry construct under constitutive promoter [22], and a helper strain *E.coli* S17-1 λpir carrying a helper plasmid (pUX-BF13) [23]. S17-1 strains additionally contain a chromosomal insertion with the conjugation elements and they were used for conjugation.

Electroporation. *E. coli* donor and helper strains were grown in 4 ml of LB supplemented with an appropriate antibiotic in 14 ml polypropylene round-bottom tubes shaken (200 rpm) at 37 °C for 24 h. Plasmids were purified using a ZR Plasmid Miniprep-Classic kit (Zymo Research) following manufacturer's instructions. Main modifications to the protocol of Choi & Schweizer [21]: (a) we harvested isolates grown in LB at OD_{600} = 0.3 - 0.7; (b) 500 ng - 1.8 µg of each plasmid were used; (c) after electroporation we recovered bacteria for 3 - 4.5 h at 28 °C shanking (160 rpm), and then (d) plated them on LB-agar (12%) with different gentamycin concentration (8, 30, 35 or 45 µg/ml), and incubated the plates at room temperature for 2 - 3 days.

Conjugation. We pelleted overnight cultures (grown as described above or in 5 ml of LB in 50 ml falcon tubes) of donor, helper, and recipient (soil or pond non-producer) at 7,500 rcf for 5 min. Then we washed the pellets with 2 ml of a 0.85% NaCl solution, pelleted at the same speed and suspended the pellets in LB broth, so that the donor and helper were 2 - 4 times more concentrated than the

recipient. A mixture of the three bacteria was incubated at 28 °C overnight as two 50 µl drops on an LB-agar (12%) plate. Afterwards, we suspended the drops in 800 µl of 0.85% NaCl solution, and spread 10 µl, 100 µl and the remaining concentrated culture on *Pseudomonas* selective plates (PIA, *Pseudomonas* isolation agar) with a proper gentamycin concentration (8, 30 or 45 µg/ml). Plates were incubated at room temperature for 2 - 4 days.

Three single colonies per isolate that were fluorescing when checked with Infinity3 camera system (Lumenera corporation), were streaked out for single colonies on LB-agar plates. We wanted to choose those transformants that grew most similar to their untagged version. For this, we first pre-grew the untagged and tagged strains in LB and then in CAA with 200 µM 2,2'-dipyridyl and compared their growth (OD_{600} after 24 h of incubation at room temperature; measured with Tecan microplate reader). The tagged strains that grew most similar to their wildtype were chosen for the next experiments.

We further wanted to check whether mCherry marker has fitness consequences for the chosen transformants (Supplementary Fig. 3). For this, we first grew untagged and tagged versions of the non-producers in LB overnight. Then we adjusted cultures to OD_{600} = 1 with LB and added 2 µl of the adjusted cultures to 200 µl of CAA with 200 µM 2,2'-dipyridyl in 96-well plates, in triplicates. OD_{600} was measured every 30 min at room temperature (25 - 28 °C) for 48 h in a Tecan microplate reader. Growth curves were analysed as described in the section 'Measurement of growth and pyoverdine production levels'. Statistical analysis revealed that, overall, mCherry marker did not impair growth of the isolates (Supplementary Fig. 3).

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Chapter 3. Project 2

‘In nature we never see anything isolated, but everything in connection with something else which is before it, beside it, under it and over it.’

~ Johann Wolfgang von Goethe

3.1 Environmental determinants of pyoverdine production, exploitation and competition in natural *Pseudomonas* communities

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Abstract

Many bacteria rely on the secretion of iron-binding siderophores to acquire this essential yet often limited element from the environment. Laboratory studies revealed that the costly production of siderophores is often fine-tuned to match prevailing levels of iron limitation which, in turn, depend on multiple abiotic environmental parameters such as iron concentration and pH. Intriguingly, the production of siderophores can also depend on biotic factors such as the presence of competing strains. This is because siderophores can be used to lock iron away from competing strains lacking a specific receptor for uptake. Conversely, the secreted molecules can become exploitable by non-producing cheaters that free-ride on the siderophores produced by others. Since little is known about whether these laboratory-derived insights are relevant in nature, we studied the production of the siderophore pyoverdine and its social effects among 930 *Pseudomonas* isolates originating from soil and pond communities. We found that pH, iron and carbon concentrations together with community diversity significantly correlated with the amount of pyoverdine natural isolates produce. Moreover, pyoverdine non-producers occurred in both habitats, with higher prevalence in soil, especially in habitats with predicted highest availability of iron. Our results suggest that in nature the evolution of non-producers can be driven not only by the opportunity to cheat, but also because iron is readily available. Finally, we found evidence for higher strain mixing in ponds evening out local variation in pyoverdine profiles, and impeding local adaptation. Our study managed to cut through the complexity of natural bacterial communities, and provides first insights into the multivariate nature of siderophore-based iron acquisition and competition among environmental pseudomonads.

Introduction

Iron is a key growth-limiting factor for most bacteria. It is required for various fundamental cellular processes, like DNA biosynthesis and respiration [1]. However, in nature iron is often limited because it is either insoluble in its ferric (Fe^{+3}) form at circumneutral pH and aerobic conditions, host-bound in the context of infections, or occurs only at very low concentrations in some habitats like the ocean [1,2]. To overcome iron limitation, many bacteria secrete siderophores, iron-scavenging molecules that have a high affinity for ferric iron [3]. These molecules bind iron from natural sources and can then be taken up by cells via specific receptors. Given the importance of iron, it comes as no surprise that bacteria have evolved sophisticated mechanisms to accurately adjust the level of siderophore production to match prevailing levels of iron limitation [1].

In addition to their role in provisioning producers with iron, siderophores can also have fitness consequences for other community members, including non-producers and cells with different siderophore systems [4–8]. This is because siderophores can be shared between individuals with compatible receptors, which can select for exploitation by cheating strains that do not produce or produce less of the specific siderophore, yet still capitalize on the siderophores produced by others [9,10]. Moreover, siderophores can lock iron away from competitors with incompatible receptors, and can therefore be involved in inter-strain competition [8,11–15]. Although laboratory studies have uncovered many of the molecular, ecological and evolutionary aspects of fine-tuned siderophore regulation and siderophore-mediated social interactions (e.g. sharing, cheating, inter-strain competition), we know surprisingly little about the drivers of siderophore production under natural conditions in complex multi-species communities.

Here, we aim to address this issue by examining how environmental factors of natural soil and pond habitats relate to the amount of siderophores produced by their inhabitants, and the ability of co-occurring natural isolates to affect each other via their siderophores. In our study, we focus on the phylogenetically diverse group of fluorescent pseudomonads, whose members are known to produce the green-fluorescent pyoverdine as their primary siderophore [16]. From laboratory studies, predominantly carried out with *P. aeruginosa*, and field studies with constructed strains, we know that iron concentration, organic carbon composition, pH, and community diversity all influence the level of pyoverdine production. Specifically, bacteria gradually downscale pyoverdine production in response to increased iron concentration [17,18], when iron is bound to relatively weak organic chelators [19], and at low pH where solubility of iron is increased [20]. In addition, *P. aeruginosa* was found to upregulate pyoverdine production in the presence of competing strains or species [18,21]. Interestingly, pyoverdine-mediated social interactions were found to be influenced by similar environmental factors. For example, increased iron availability allows producers to down-scale pyoverdine production, which lowers their susceptibility to exploitation by non-producers [22]. Similarly, increased carbon availability

reduces the relative metabolic costs of pyoverdine production, and thus decreases the advantage of cheaters [23,24]. Finally, community composition was found to be a key factor determining who interacts with whom, thereby influencing the relative importance of competition versus cooperation [25–27].

To examine whether these factors are also associated with patterns of pyoverdine production and pyoverdine-mediated social interactions in natural communities, we isolated a total of 930 pseudomonads from 24 soil and 24 pond communities. We measured pyoverdine production capacities of all isolates in a standard iron-limited medium, and related these measures to the pH, the total iron and carbon concentrations of their environment, and the phylogenetic diversity of their community. For a subset of strains and communities, we further quantified the effect of pyoverdine-containing supernatant on the fitness of co-occurring strains. For these experiments, we focused on pyoverdine non-producers because growth stimulation and inhibition by foreign pyoverdines provide information on whether strains possess matching receptors for the social exploitation of heterologous siderophores [8]. We then related these social parameters to the phylogenetic relatedness between strains and the environmental parameters described above.

Results

Pyoverdine production profiles differ between soil and pond pseudomonads

Prior to phenotypic screening, we sequenced the housekeeping *rpoD* gene, commonly used for phylogenetic affiliation of pseudomonads, for all 930 isolates to confirm that they indeed belong to this taxonomic group (Fig. S1). Once this was confirmed, we measured the pyoverdine production abilities of all isolates (using the natural fluorescence of this molecule) in iron-limited casamino acids (CAA) medium supplemented with transferrin as an iron chelator. We found that isolates greatly varied in the amount of pyoverdine they make (Fig. 1A). While some strains produced no measurable amount of pyoverdine, others produced much more than our laboratory reference strains (pyoverdine values are scaled relative to the average production level of the laboratory reference strains listed in Table S1). Across all isolates, there was a positive correlation between overall pyoverdine levels and their growth in iron-limited CAA, suggesting that pyoverdine is important to overcome iron limitation (linear mixed model, LMM: $t_{917.7} = 18.62$, $p < 0.001$; Fig. 1B).

There were several significant differences between soil and pond isolates. First, the relative pyoverdine production was significantly higher for pond than for soil isolates (Fig. 1A, mean ± SE for pond isolates: 0.832 ± 0.018; for soil isolates: 0.571 ± 0.022; LMM: $t_{21.5} = 4.36$, $p < 0.001$). Second, there were significantly more pyoverdine non-producers present in soil (19.7%, defined as producing less than 5% of laboratory reference strains) than in pond (8.3%) communities (Fisher's exact test: $p <$

0.0001). Finally, the coefficient of variation (CV) in relative pyoverdine production among isolates from the same community was significantly lower in pond than in soil communities (mean CV ± SE for pond communities: 46.2 ± 2.4%; for soil communities: 85.2 ± 8.3%; LMM: $t_{22.0} = -4.39$, $p < 0.001$; Fig. S2).

To explore the relationship between phylogenetic diversity and pyoverdine production, we constructed maximum likelihood phylogenetic trees based on partial *rpoD* gene sequences for pond and soil isolates (Fig. S1). We found that phylogenetic diversity (normalized by the number of isolates per community) was significantly higher for pond than soil communities (Faith's phylogenetic diversity, median ± [1st quartile | 3rd quartile], for pond communities: 0.11 ± [0.09 | 0.15]; for soil communities: 0.09 ± [0.07 | 0.11]; Mann-Whitney U-test: $W = 178$, $p = 0.023$).

We further examined whether there is a phylogenetic signal for pyoverdine production (i.e. whether closely related isolates show similar pyoverdine production) (Fig. S1). Compatible with our previous findings [8] (on a smaller data set), we found a weak phylogenetic signal for pond communities (Blomberg's $K = 0.12 \pm [0.03 \mid 0.25]$), and an even lower phylogenetic signal in soil communities ($K = 0.02 \pm [0.01 \mid 0.12]$; test for differences between pond and soil communities, Wilcoxon rank sum test: $W = 186$, $p = 0.036$). These analyses confirmed that pyoverdine production levels can highly vary even among closely related strains.

Environmental determinants of pyoverdine production

Next, we tested for a relationship between the pyoverdine production profiles described above and different abiotic (pH, total iron and carbon concentrations) and biotic (phylogenetic community diversity) variables of the environments the isolates originated from. These environmental variables varied considerably between communities (Table S2), and were often correlated across communities (Table S3). To account for the resulting collinearities, we first carried out separate principal component analyses (PCAs) for soil and pond that each incorporated the four environmental variables. PCAs break collinearities by converting the values of potentially correlated variables into a set of uncorrelated variables called principal components (PCs). Here, we focused on the first two PCs (henceforth called SPC1 and SPC2 for soil, and PPC1 and PPC2 for pond), which explained 88.0% (soil) and 88.3% (pond) of the total variance observed (Table 1). Each PC is characterized by a set of positive and/or negative loadings, which describe how much it is influenced by each of the environmental variables fed into the PCA. These PCs were then used in standard linear models to test whether they correlate with the pyoverdine production levels of our isolates.

For soil communities, SPC1 was positively loaded by carbon concentration, and negatively by pH and iron concentration, whereas SPC2 was positively loaded by community diversity (Table 1A).

Opposing signs of loadings, as occurring for SPC1 (carbon vs. pH/iron) indicate trade-offs (i.e. negative correlations) between the factors involved. When relating these PCs to the pyoverdine production profiles of the soil isolates, we found a positive correlation between relative pyoverdine production and community diversity (SPC2; $t_{18.3} = 3.36$, $p = 0.0034$; Fig. 2A). In contrast, there was no significant association between relative pyoverdine production and the trade-off between carbon versus pH and iron captured by SPC1 ($t_{10.6} = -1.93$, $p = 0.0809$). Because there were many pyoverdine non-producers, present in 22 out of 24 soil communities, we further examined whether the likelihood of being a pyoverdine non-producer correlates with the SPCs. We found that the likelihood of being a non-producer was highest in communities where relatively high levels of carbon occurred in combination with relatively low levels of pH and iron (SPC1; $z = 2.85$, $p = 0.0044$; Fig. 2B). Conversely, the likelihood of being a non-producer did not correlate with community diversity (SPC2; $z = -0.72$, $p = 0.4745$).

For pond communities, PPC1 was positively loaded by iron and carbon, and negatively by pH and community diversity, representing a trade-off between these groups of environmental factors (Table 1B). Conversely, PPC2 was positively loaded by pH and carbon. Note that this positive association is independent of and occurs simultaneously with the trade-off between these variables in PPC1 (Table 1B). When feeding these PCs into a linear mixed model we found a significant negative association between relative pyoverdine production and PPC2 ($t_{10.9} = -2.92$, $p = 0.0140$), indicating that the relative pyoverdine production was lower among pond isolates from communities characterized by higher levels of carbon and pH (Fig. 2C). In contrast, PPC1 was not linked to the pyoverdine production of pond isolates ($t_{10.0} = 0.68$, $p = 0.5120$). We further examined whether the likelihood of being a pyoverdine non-producer correlated with one of the PPCs, but this was neither the case for PPC1 ($z = -1.16$, $p = 0.2450$) nor for PPC2 ($z = 0.79$, $p = 0.4320$).

The genetic basis of pyoverdine non-production

The occurrence of many pyoverdine non-producers was unexpected, and prompted us to explore their genetic makeup in more detail. Our previous study [8] entailing whole-genome sequencing of environmental isolates revealed that two different types of non-producers exist: those with a highly truncated pyoverdine locus differed from the ones having a seemingly intact, yet largely silent locus. To find out whether this pattern also applies here, and whether the frequency of the two types correlates with environmental variables, we screened all 130 non-producers for the presence of the *pvdL* gene. This gene encodes an essential and conserved non-ribosomal peptide synthetase involved in pyoverdine synthesis [28,29]. The presence or absence of the *pvdL* gene would indicate whether non-producers possess a silent yet intact locus or a truncated dysfunctional locus, respectively.

We found that 32 out of 91 (35.2%) soil non-producers and 15 out of 39 (38.5%) pond non-producers were positive for the presence of *pvdL* (no significant difference between soil and pond: $\chi^2 = 2.48$, $p = 0.1151$). Consistent with our previous results [8] *pvdL*-positive strains could produce residual amounts of pyoverdine (mean relative pyoverdine production ± SE, 0.014 ± 0.002, relative to laboratory reference strains), whereas virtually no pyoverdine fluorescence signal was detected in *pvdL*-negative strains (mean ± SE, 0.0003 ± 0.0001) (Fig. S3). For soil communities, the likelihood of being *pvdL*-positive was highest in communities characterized by the combination of low carbon, high iron concentrations and high pH (SPC1: $z = -2.07$, $p = 0.0386$; Fig. 2D). For pond communities, meanwhile, none of the two PPCs were significantly associated with the likelihood of being *pvdL*-positive (PPC1: $z = -1.54$, $p = 0.1230$; PPC2: $z = -0.83$, $p = 0.4090$).

Supernatants from pyoverdine producers affect the growth of non-producers

To estimate the extent to which pyoverdine could be involved in social interactions between strains, we compared growth of non-producers in iron-limited media supplemented with or without pyoverdine-containing supernatants of producers from the same community. Overall, we fed 53 non-producers (from 12 pond and soil communities each) with pyoverdine-containing supernatants from four to six different producers from the same community. This resulted in a total of 151 pond and 152 soil non-producer-supernatant combinations. The growth data from the supernatant assay was then used to calculate the likelihood of a non-producer to be stimulated or inhibited by a supernatant.

We found that the fitness effects of pyoverdine-containing supernatants on non-producers covered the entire range from almost complete growth inhibition to high stimulation (Fig. S4). While the likelihood of stimulation did not significantly differ between the two habitats (soil vs. pond: $z = 1.80$, $p = 0.072$; Fig. 3A), the likelihood of inhibition was significantly higher for pond than for soil non-producers ($z = 2.86$, $p = 0.004$; Fig. 3B). Note that ‘stimulation’ or 'inhibition' describe the respective cases where non-producers grew significantly better or worse, with pyoverdine-containing supernatants than without. Although supernatants contain other growth-modulating components in addition to pyoverdine, we have previously shown that the growth effects of this supernatant assay on non-producers are mainly caused by pyoverdine, and are likely driven by the presence (stimulation) and absence (inhibition) of a matching receptor for pyoverdine uptake [8].

Environmental determinants of pyoverdine-mediated social interactions

Next, we tested whether the likelihood of stimulation and inhibition correlate with environmental factors. To this end, we reused the first two PCs describing the relationships between our four environmental variables of interest (pH, iron and carbon concentrations, community diversity) as explanatory variables. Because the supernatant assay involved pairs of strains (i.e. the supernatant

donor and the recipient), we further included the phylogenetic relatedness between isolates, based on *rpoD* sequence similarity, into our statistical models.

For soil communities, we found that the likelihood of stimulation correlated with SPC1, suggesting that non-producers were most likely to be stimulated by heterologous pyoverdine when originating from environments featuring a combination of a high carbon concentration on the one hand, and a low pH and iron concentration on the other hand (z = 2.21, p = 0.0269; Fig. 4A). Furthermore, the likelihood of stimulation tended to increase with community diversity (SPC2; z = 1.96, p = 0.0502), and increased with the relatedness between strains (*rpoD* identity; z = 3.44, p = 0.0006; Fig. 4B). The opposite trends were generally observed for the likelihood of inhibition: it correlated negatively with SPC1 (z = -1.94, p = 0.0525), and relatedness (*rpoD* identity; z = -2.80, p = 0.0052), but not with community diversity (SPC2; z = -1.01, p = 0.3144).

For pond communities, we observed that the likelihood of stimulation negatively correlated with PPC1, suggesting that non-producers were most likely to be stimulated by heterologous pyoverdine(s) produced by other community members when originating from environments featuring low levels of carbon and iron together with high pH and community diversity (z = -2.13, p = 0.0333; Fig. 4C). Moreover, the likelihood of stimulation depended on an interaction between PPC2 and the phylogenetic relatedness between the supernatant donor and the recipient (interaction: z = 2.72, p = 0.0066; main effects: *rpoD* identity; z = 1.31, p = 0.1900; and PPC2; z = 0.65, p = 0.5132). In particular, a relatively high phylogenetic relatedness increased the likelihood of stimulation in communities featuring high levels of PPC2 (i.e. simultaneously high levels of pH and carbon), whereas this relationship was reversed in communities featuring low levels of PPC2 (Fig. 4D). By contrast, neither the PPCs nor *rpoD* identity correlated with the likelihood of inhibition (PPC1: z = 1.82, p = 0.0688; PPC2: z = -1.30, p = 0.1943; *rpoD* identity: z = -1.37, p = 0.1701).

Discussion

The beauty of laboratory experiments in microbiology is that factors influencing bacterial physiology, behaviour, and fitness can be investigated one at the time under controlled and replicable conditions. This approach contrasts with the situation bacteria typically face in nature, where environmental conditions often fluctuate rapidly in unpredictable manners, with multiple variables simultaneously influencing bacterial behaviour and fitness. This raises the question whether the factors affecting bacterial behaviour in vitro also play a role under natural conditions. Our study tackled this question by examining whether factors shown to influence an important bacterial trait in the laboratory, the production of siderophores used for iron-scavenging, also affect this behaviour in natural communities. As a model system, we focused on the siderophore pyoverdine produced by

Pseudomonas bacteria. Our investigations involving 930 *Pseudomonas* isolates, originating from 24 soil and 24 pond communities, yielded several novel insights. First, we found that pH, concentrations of total iron and carbon, and community diversity, all shown to be important determinants of pyoverdine production in the laboratory, are indeed correlated with the level of pyoverdine produced by natural *Pseudomonas* isolates. Second, we observed that the same environmental variables also correlated with pyoverdine-mediated social interactions, measured by the extent to which secreted pyoverdine could promote or inhibit the growth of other members of the community. Third, we showed that the way these environmental factors correlated with pyoverdine production and social interactions differed fundamentally between soil and pond communities. Finally, our data suggest that trade-offs and interactions between environmental factors, which are typically ruled out in the laboratory, could be more predictive of bacterial behaviour in nature than the main effect of a single factor.

We observed that isolates from soil and pond communities differ substantially in their pyoverdine production profiles and in the way their production levels seem to be influenced by environmental factors. In particular, we found that: (a) soil isolates produced on average significantly less pyoverdine than pond isolates (Fig. 1A); (b) there were significantly more pyoverdine non-producers in soil than in pond (Fig. 1A; Table S2); and (c) the four environmental variables examined (community diversity, pH, total iron and carbon concentrations) were all associated with at least one aspect of pyoverdine production for soil isolates, which was not the case for pond isolates, whose pyoverdine production seemed to be much less affected by these variables (Fig. 2). One obvious reason for these differences is that soils and ponds differ substantially in many characteristics. For instance, pH variation was greater among soil than pond communities, and iron and carbon concentrations were orders of magnitude higher in soils compared to ponds (Table S2). Moreover, the overall spatial structure is conceivably higher in soil than in pond communities, potentially promoting strain mixing in the latter – a hypothesis that is supported by our finding that community diversity was significantly higher in ponds compared to soils. Given these fundamental differences, the observation that pond isolates make more pyoverdine could simply be interpreted as an evolutionary response to the more stringent iron limitation in this habitat. Furthermore, low spatial structure and increased strain mixing might even out local variation in community composition and hamper local adaptation, explaining why environmental factors are worse predictors of pyoverdine production in ponds than soils.

In contrast to the higher pyoverdine production among pond isolates, the observation that there were more pyoverdine non-producers in soils than in ponds is not so straightforward to explain (Fig. 1A). We initially expected the opposite pattern because laboratory studies showed that non-producers are successful in cheating when strain mixing is high, iron limitation is severe and carbon availability is low [18,23,24], exactly the conditions prevailing in pond communities. So, if non-producers are cheaters, why were they not more prevalent in ponds? One explanation could be that bacterial density is

generally low in aquatic systems, which could help to keep cheaters at bay. Indeed, previous studies showed that successful cheating is often only possible at high cell density, where non-producers are close to producers, and thus can benefit from the secreted public goods [7,30–32]. These conditions are more likely to be met in soil communities, where high nutrient availability can support high cell densities, which can in turn favour cheating, especially among closely related strains (Fig. 4B).

An alternative explanation for the higher prevalence of non-producers in soils compared to ponds is that soil non-producers might not all have evolved because of cheating, but lost the ability to make pyoverdine due to other reasons. It is indeed intriguing that the likelihood of being a non-producer was highest in soils characterized by a high carbon concentration in combination with low pH/iron. These are the conditions where we predict the highest iron bioavailability because low pH is considered to be the main factor increasing the bioavailability of iron (and other metals) in soil [33,34]. In addition, a high carbon concentration is not only known to decrease the cost of pyoverdine production [23,24], but can also increase the bioavailability of iron, via the metal complexing properties of organic compounds, such as humic and fulvic acids [35–39]. Based on these considerations, one could thus conclude that soil non-producers arise under conditions where pyoverdine is redundant and not needed for growth. If this was indeed the case, why did non-producers not fix under these conditions, but always co-occurred with producers? And why was the likelihood of non-producers being stimulated by pyoverdine-containing supernatant highest under exactly these conditions (high carbon in combination with low pH/iron), where non-producer prevalence was highest? These two findings speak against the hypothesis that pyoverdine is completely redundant, but rather suggest that pyoverdine production and exploitation are also important under conditions of increased iron bioavailability.

It is known that siderophores might be deployed as agents to inhibit competitors [11,13–15,40]. This is because siderophores bind iron in the environment, thereby locking it away for competitors lacking a matching receptor for uptake. Consequently, bacteria are predicted to upscale siderophore investment with increased levels of competition [21,41]. In support of this hypothesis, we found that soil isolates make more pyoverdine in more diverse *Pseudomonas* communities (i.e. a proxy for the level of inter-strain competition for iron) (Fig. 2A). Inhibition via siderophores was suggested to be successful even when iron is relatively abundant, as siderophore production induces artificial iron limitation for competitors [14]. Iron-replete conditions could still favour pyoverdine non-producers, because pyoverdine is not stringently needed when iron is relatively abundant. However, presence of inhibitory pyoverdines in the environment would then be expected to select for the preservation (or even the additional accumulation) of an array of pyoverdine receptors, which prevents non-producers from being inhibited by pyoverdine-producing competitors [14]. This scenario is compatible with our observation that the prevalence of non-producers (Fig. 2B) and the likelihood of stimulation (Fig. 4A) was increased in soil communities with highest predicted iron availability. Interestingly, many non-producers in these communities showed evidence for a highly degraded pyoverdine locus, as most of

them were *pvdL*-negative (Fig. 2D). This indicates that a permanent trait loss is more common under conditions of relatively high iron bioavailability, which contrasts with the presence of a silent yet intact locus prevalent in many of the other non-producers.

Finally, we now turn to pond communities and ask how environmental factors might influence pyoverdine production profiles and social interactions in these aquatic habitats. As already discussed above, we propose that the openness of aquatic systems, characterized by increased strain influx from surrounding terrestrial habitats and increased strain mixing, might reduce the potential for local strain adaptation typically seen in soil [43]. Still, we observed that some environmental variables were associated with both pyoverdine production levels of isolates (Fig. 2C) and social interactions between them (Fig. 4C+D). These patterns are yet less intuitive to explain than those found among soil isolates. In any case, we predict that open systems are more prone to random interactions between strains, and several of our results can indeed be interpreted in this light. For instance, random interactions between pyoverdine producers and non-producers should reduce the probability of non-producers coincidently possessing a matching receptor for a specific pyoverdine. This should lead to increased levels of pyoverdine-mediated growth inhibition, which we indeed observed in our pond communities (Fig. 3B). In contrast, the likelihood of stimulation could be higher in more diverse communities, simply because the probability for having a strain with a compatible pyoverdine in the system is increased (as shown in Fig. 4C). Nonetheless, we presume that local adaptation is still possible in such open systems. For instance, we predict that random strain encounters in the dilute and iron-limited environments like ponds might force non-producers and producers to acquire multiple different receptors to be able to 'take a bit from everybody'. Indeed, when reanalysing data from our previous study [8], where we sequenced the genomes of 24 isolates (also featured in this study), we found that pond isolates possessed significantly more pyoverdine receptor homologues in their genomes compared to soil isolates (median no. of receptor homologues ± [1st quartile | 3rd quartile] for soil isolates: 2.5 [2 | 3.5], for pond isolates: 5.5 [3.75 | 6.67]; Wilcoxon rank sum test: $W = 33$, $p = 0.0243$). These findings suggest that selection pressures with regard to pyoverdine-mediated social interactions differ fundamentally between soil and freshwater habitats.

In conclusion, our study reveals that connecting laboratory to field studies is a challenging task for microbiologists. While laboratory studies are typically carried out with isogenic strains and engineered single-gene knockout mutants grown under controlled conditions, field studies like ours face an enormous strain diversity, and a plethora of environmental factors that all simultaneously vary and influence bacterial behaviour. Despite this complexity, we managed to obtain first insights into the importance of siderophore-based iron acquisition and competition in natural communities of pseudomonads. In particular, we found evidence that pyoverdine production levels of natural isolates and the ability to use heterologous pyoverdine are simultaneously influenced by multiple environmental variables, including pH, iron and carbon concentrations, with patterns clearly differing

between soil and pond habitats. Moreover, we found that pyoverdine non-producers occurred in both habitat types, and many of them have the ability to take up heterologous pyoverdines, and probably evolved as cheaters exploiting producers, or because pyoverdine is not necessarily needed for growth. We hope our work spurs future studies that embrace the complexity of natural systems, in order to learn more about microbial behaviour and strain interaction patterns, and how they affect community composition and population dynamics.

Methods

Sampling and isolation of pseudomonads. We sampled eight *Pseudomonas* communities at each of three pond and three soil locations (48 communities in total). While one pond and one soil location were situated on the campus of the University of Zurich Irchel (47.40° N, 8.54° E), the other two soil and two pond habitats were located at Seleger Moor Park (47.25° N, 8.51° E), Switzerland. The a priori reasoning for sampling at these different locations was that we expected ecological parameters to differ quite substantially between the habitats in a city park and a more natural environment. We sampled soil cores and pond water, isolated 952 strains on the medium selective for fluorescent pseudomonads from these samples, and preserved the isolates as freezer stocks as described in detail elsewhere [8]. We provided each isolate with a unique identification code, consisting of a location ID ('s1 = Seleger Moor soil#1', 's2 = Seleger Moor soil#2', 's3 = Irchel soil', '1 = Seleger Moor pond#1', '2 = Seleger Moor pond#2', '3 = Irchel pond'), a community ID (small letters 'a' to 'h for soil communities; capital letters 'A' to 'H' for pond communities) and an isolate number (1 to 20).

Evaluation of environmental parameters. We measured the pH of soil and pond samples using the Atago portable pH meter DPH-2 (for field measurements) and the Metrohm 744 pH meter (for laboratory measurements). The pH of pond samples was first measured in the laboratory and then later confirmed by measurements directly in the field. Prior to soil pH measurements, the soil samples were suspended 1:5 (w/v) in 0.01 M $CaCl_2$ solution, shaken for 1 h, and left to sediment.

We measured the percentage of carbon in soil and pond samples with a CHN (Leco Truspec micro; Leco Instruments, USA) and a TOC analyser (Dimatoc 2000; Dimatec, Germany), respectively. Measurements were carried out by staff operating the specific in-house service at the University of Zurich. Prior to the measurements, we first dried soil samples for two days at 40 °C, then grounded them to a homogenous powder and dried them again at 40 °C overnight.

To estimate the iron concentration of the sampled soils and ponds, ICP-OES (Vista-MPX, Varian, USA) was used. For soils, we first dried and homogenized the plant-free samples (plant material was removed using a 0.6 mm stainless steel sieve). Soil samples were then digested with HCl and HNO_3

for 90 min at 120 °C, diluted in Nanopure water and filtered. The pond samples were directly filtered using a 0.2 µm filter and then acidified with 65% HNO_3 to pH < 3. To make iron concentrations comparable between soil (µg/g) and pond (µg/L) samples, we converted pond concentrations to µg/g by assuming that 1 ml = 1 g.

***rpoD* amplification and sequencing.** To verify that the 952 isolates are indeed *Pseudomonas*, we PCR amplified and sequenced a part of the *rpoD* gene for 930 isolates (PCR or sequencing failed for 22 isolates, which were excluded from further experiments). This housekeeping gene is commonly used for phylogenetic affiliation of pseudomonads [44,45]. PCR mixtures were prepared, PCR reactions were carried out and the products were sequenced using PsEG30F and PsEG790R primers [44] as described elsewhere [8].

Community diversity and phylogenetic relatedness. A codon-aware nucleotide alignment of *rpoD* was generated using local TranslatorX v1.1 [46] with the MAFFT v7.271 [47] aligner. We manually curated and trimmed the alignment at both ends resulting in a high-quality alignment of 908 sequences (including 21 reference strains; as described elsewhere [8]) over 609 nucleotides. The phylogenetic tree was inferred by RAxML v7.0.4 [48] using the General Time Reversible (GTR) + G model with 100 bootstraps. We calculated phylogenetic diversity within communities based on the cophenetic function from the ape package in R software and normalized values by the number of tips. For some analyses, we calculated the relatedness between strains by carrying out pairwise alignments of *rpoD* sequences using the 'water' application from EMBOSS [49].

***pvdL* amplification.** We used the presence of the *pvdL* gene as a proxy for the presence of the pyoverdine biosynthesis locus in all 130 pyoverdine non-producers. *pvdL* is involved in the biosynthesis of the pyoverdine chromophore, and is the most conserved part of the locus across different pseudomonads [28]. To identify a conserved region of this gene, we aligned the *pvdL* sequences (retrieved from GenBank) of seven pseudomonads: *P. aeruginosa* PAO1, *P. chlororaphis* O6, *P. chlororaphis subsp.aureofaciens* 30-84, *P. fluorescens* A506, *P. fluorescens* SS101, *P. protegens* Pf-5, *P. syringae pv.syringae* B64. We then designed the following primers to amplify a conserved region in the isolated non-producers (pvdL_fw: CATGATGAGCAACCACCACATC, pvdL_rv: CGCTGGTCGTAGGACAGGTG; product size: 827 bp). We prepared PCR mixtures as for the *rpoD* gene amplification [8], just this time we used a 'Hot Start' version of the Master Mix. Bacterial biomass was taken from fresh cultures grown in liquid LB medium (1 µl). We used the following PCR conditions: denaturation at 94.5 °C for 5 min; 30 cycles of amplification (1 min denaturation at 94 °C, 1 min primer annealing at 57 °C, and 1 min primer extension at 68 °C); final elongation at 68 °C for 10 min. As a positive control, we used 72 pyoverdine producers (pond and soil, 36 each). We considered an isolate as *pvdL*-positive when the PCR yielded a product of the expected size. Among the 72 pyoverdine producers (which should all have *pvdL*), we found seven to be negative for *pvdL*.

We thus estimate the rate of false-negatives to be 9.7% (i.e. the risk to falsely identify isolates as *pvdL*-negatives).

Measurement of growth and pyoverdine production levels. To evaluate whether and to what extent the isolates can produce pyoverdine, we grew them under iron-limited conditions and measured their pyoverdine production levels. We first grew the isolates in 150 µl LB in 96-well plates overnight (16 - 18 h) static at room temperature. We then transferred 2 µl of overnight cultures to 200 µl iron-limited casamino acids medium (CAA containing 5 g casamino acids, 1.18 g $K_2HPO_4 \cdot 3H_2O$, 0.25 g $MgSO_4 \cdot 7H_2O$ per liter) supplemented with 25 mM HEPES buffer, 20 mM $NaHCO_3$ and 100 µg/ml of the strong iron chelator human apo-transferrin in a 96-well plate. All chemicals were purchased from Sigma-Aldrich, Switzerland.

Each plate had isolates from one community in triplicates and eight reference strains known to produce pyoverdine (see Table S1 for a description of these strains). After 18 h of incubation at room temperature, we measured growth (optical density OD at 600 nm) and pyoverdine production levels (relative fluorescence units, RFU, with excitation: 400 nm and emission: 460 nm) with an Infinite M200 Pro microplate reader (Tecan Group Ltd., Switzerland). We then scaled the growth and relative pyoverdine production values for each isolate by dividing their OD_{600} and RFU by the average respective OD_{600} and RFU of the reference strains.

We also carried out a control experiment to verify that it is indeed iron limitation that induces the observed growth and pyoverdine production patterns, and not a specific effect of transferrin as an iron chelator. For this, we repeated the growth assay with a synthetic iron chelator 2,2'-dipyridyl (200 µM). The growth assay was carried out for a subset of soil (n = 100: 28 pyoverdine non-producers and 72 producers) and pond isolates (n = 99: 27 non-producers and 72 producers), from 12 soil and 12 pond communities, in three replicates. The assay yielded qualitative very similar results for both iron chelators (Fig. S5). Moreover, in an earlier study we could demonstrate that the environmental isolates (n = 315) could use CAA as a carbon source, demonstrating that the poor growth of non-producers is due to their inability to produce pyoverdine, and not because they are unable to grow in CAA [8].

Supernatant assay. To quantify the effect that pyoverdine-containing supernatants from producers have on non-producers, we set up a supernatant growth assay using a subset of isolates and communities (24 communities in total: four communities per location and habitat type). For each community, we first selected six pyoverdine producers. To be considered for the supernatant assays, producers had to: (a) grow better than the corresponding non-producers under iron-limited conditions; and (b) differ in their *rpoD* sequence, and thus represent phylogenetically different strains. These criteria were met for 22 communities. For the two remaining communities (3E and s2e), we picked two producers with identical *rpoD* sequences, whereby for s2e we were limited to four producers satisfying (a). For each community, we further randomly selected up to three non-producers (upon

availability). In total, we had 26 and 27 non-producers (relative pyoverdine production < 0.05) from soil and pond communities, respectively. This resulted in 152 soil non-producer-supernatant and 151 pond non-producer-supernatant combinations.

To generate pyoverdine-containing supernatants, we first grew the producers (n = 142) in 200 µl LB medium in 96-well plates overnight at 25 °C. Then, 20 µl of overnight cultures were added to 2 ml of CAA with 200 µM 2,2'-dipyridyl in 24-well plates and incubated static at 25 °C for about 22 h. We centrifuged cultures for 10 min at 3,500 rpm (Eppendorf Centrifuge 5804R) and then transferred 900 µl of supernatants to PALL AcroPrep Advance 96-well 1 ml filterplates (with 0.2 µm supor membrane), attached to an autoclaved 1.2 ml 96-well PCR plate (VWR). We centrifuged the assemblies of filter and collection plates at 2,500 rpm for 15 min. The collection plates with sterile supernatants and blank medium were sealed with Greiner SILVERseal and kept at -20 °C.

Afterwards we grew the non-producers in 200 µl of LB in 96-well plates overnight static at 25 °C. We then added 2 µl of non-producer cultures to CAA with 200 µM 2,2'-dipyridyl supplemented with 20 µl of (a) producer supernatant or (b) CAA with 200 µM 2,2'-dipyridyl that went through the same treatment as supernatants, i.e. filtering and freezing. Total culturing volume was 200 µl. Each treatment was set up in four replicates. Plates were incubated static for 17 h at 25 °C. The final OD_{600} of the cultures was measured using the Tecan microplate reader. We considered a supernatant effect as 'stimulation' or 'inhibition' when non-producers grew significantly better or worse, respectively, with a supernatant than without, based on a Wilcoxon test.

Statistical analysis. We used linear mixed-effects (LMM) and linear generalized mixed-effects (GLMM) models for statistical data analysis. Since strains isolated from the same and close communities (50 cm apart; we called neighbouring communities a 'core') might not be independent from one another, we included core and community identities as random factors into our models. Whenever appropriate, we log-transformed (base e) data to meet the assumption of normally distributed residuals. All statistical analyses were carried out using the R 3.1.2 statistics software (www.r-project.org).

The environmental variables were often highly correlated (Table S3), which led to high collinearities in statistical models (variance inflation factor (vif) >> 10). We thus conducted principal component analyses (PCAs) to obtain non-correlated principal components (PCs) reflecting single or combinations of different environmental variables (entered after centering and scaling to unit variance, respectively). We performed two separate PCAs for soil and pond communities, because the overall differences in pH and the total concentrations of iron and carbon (Table S2), together with differences in the signs of some of the correlations among environmental variables in the soil and pond (Table S3), also lead to high collinearity (variance inflation factor >> 10) in models deploying PCs based on

the whole dataset. We thus used the PCs resulting from separate PCAs for soil and pond (Table 1) to analyse their effects on pyoverdine production and social interactions.

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Author contribution. EB and RK designed the study, EB isolated the strains and performed the experiments, EB, JK, SW and RK analysed the data and wrote the paper.

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Figures

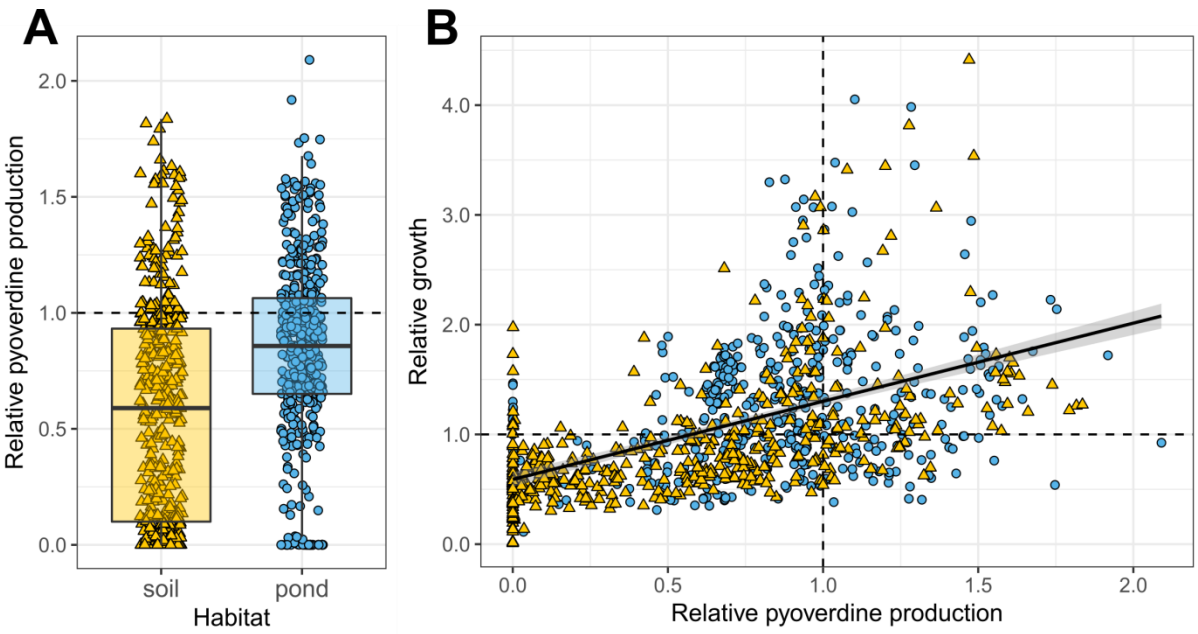


Figure 1. Environmental pseudomonads vary greatly in their level of pyoverdine production and ability to grow under iron-limited conditions. (A) Our phenotypic screen involving 930 natural pseudomonads from 24 soil and 24 pond communities (*n* = 462 soil and *n* = 468 pond) revealed that soil isolates produce significantly less pyoverdine than pond isolates, and that there are significantly more non-producers in soil than in pond communities. **(B)** There is a positive correlation between the growth (optical density measured at 600 nm) of isolates and their pyoverdine production level, indicating that pyoverdine is important to cope with iron limitation. Pyoverdine production and growth were measured in iron-limited CAA medium, and scaled relative to the values of characterized laboratory reference strains (Table S1). Values represent means across three replicates for soil (yellow triangles) and pond (blue circles) isolates. Dashed lines indicate pyoverdine production (horizontal line in A, vertical line in B) and growth (horizontal line in B) of the reference strains. Box plots show the median (bold line), the 1st and 3rd quartile (box), and the 5th and 95th percentile (whiskers).

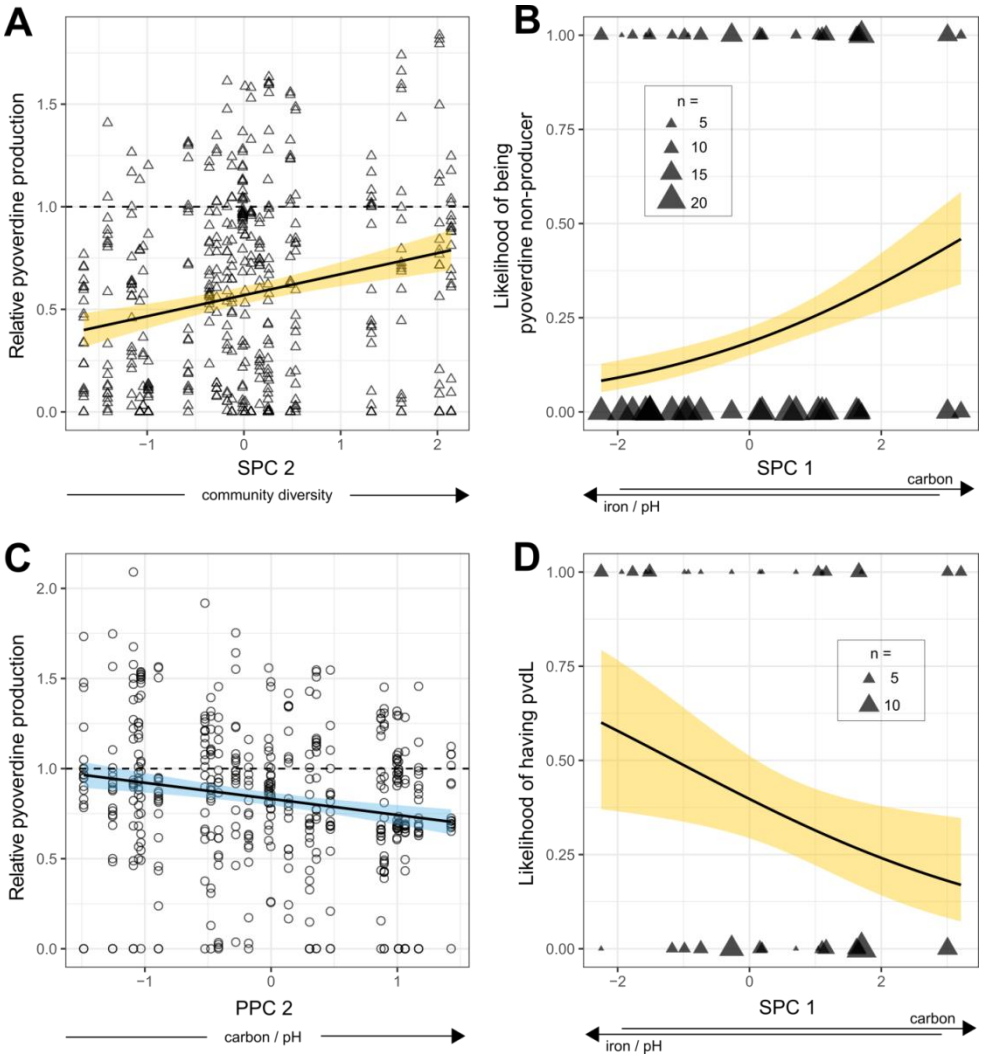


Figure 2. Correlations between parameters of pyoverdine production and environmental variables. In soil communities (*n* = 462 isolates), relative pyoverdine production in iron-limited medium increased with community diversity (SPC2) **(A)**, whereas the likelihood of being a pyoverdine non-producer correlated with SPC1, i.e. increased with higher concentration of carbon in combination with lower pH and reduced iron concentration **(B)**. For pond communities (*n* = 468 isolates), relative pyoverdine production correlated negatively with PPC2, i.e. decreased with higher concentrations of of carbon in combination with increased pH **(C)**. A PCR amplification of the gene *pvdL* in the 91 non-producers from soil revealed a negative correlation between the likelihood of having *pvdL* and SPC1 **(D)**. Since *pvdL* serves as an indicator for the presence of the pyoverdine biosynthesis locus, this analysis indicates that the likelihood of still having *pvdL*, although being a non-producer, increases in habitats characterized by high pH and iron in combination with low carbon. Solid lines with shaded areas show significant correlations together with the 95% confidence band for soil (yellow) and pond (blue) communities. Dashed lines depict scaled pyoverdine production of reference strains (Table S1).

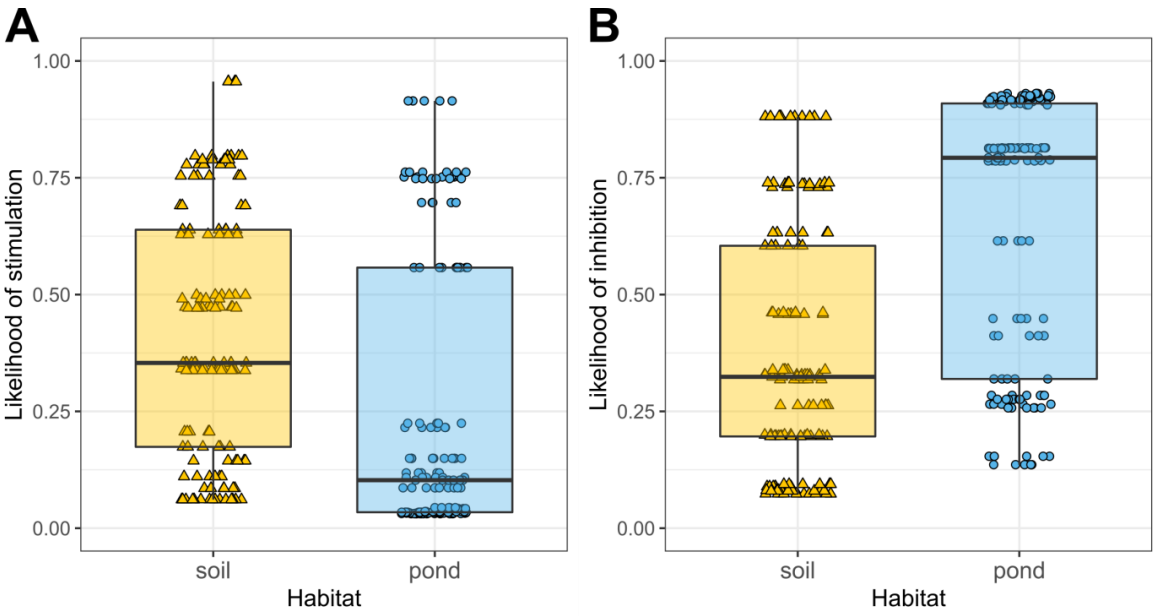


Figure 3. Pyoverdine-containing supernatants from producers can both stimulate and inhibit the growth of non-producers. (A) The likelihood for non-producers to be stimulated by pyoverdine-containing supernatants from a producer did not significantly differ between pond and soil isolates. **(B)** In contrast, the likelihood for non-producers to be inhibited by producer supernatants was significantly higher for pond than for soil isolates. The supernatant assay involved 53 (soil 26 / pond 27) non-producers, and 142 (70/72) producer supernatants from a subset of communities (12/12), resulting in a total of 303 (152/151) non-producer-supernatant combinations, each replicated three to four times. 'Stimulation' or 'inhibition' corresponded to cases where non-producers grew significantly better or worse with supernatant than without, respectively. Box plots show the median (bold line), the 1st and 3rd quartile (box), and the 5th and 95th percentile (whiskers).

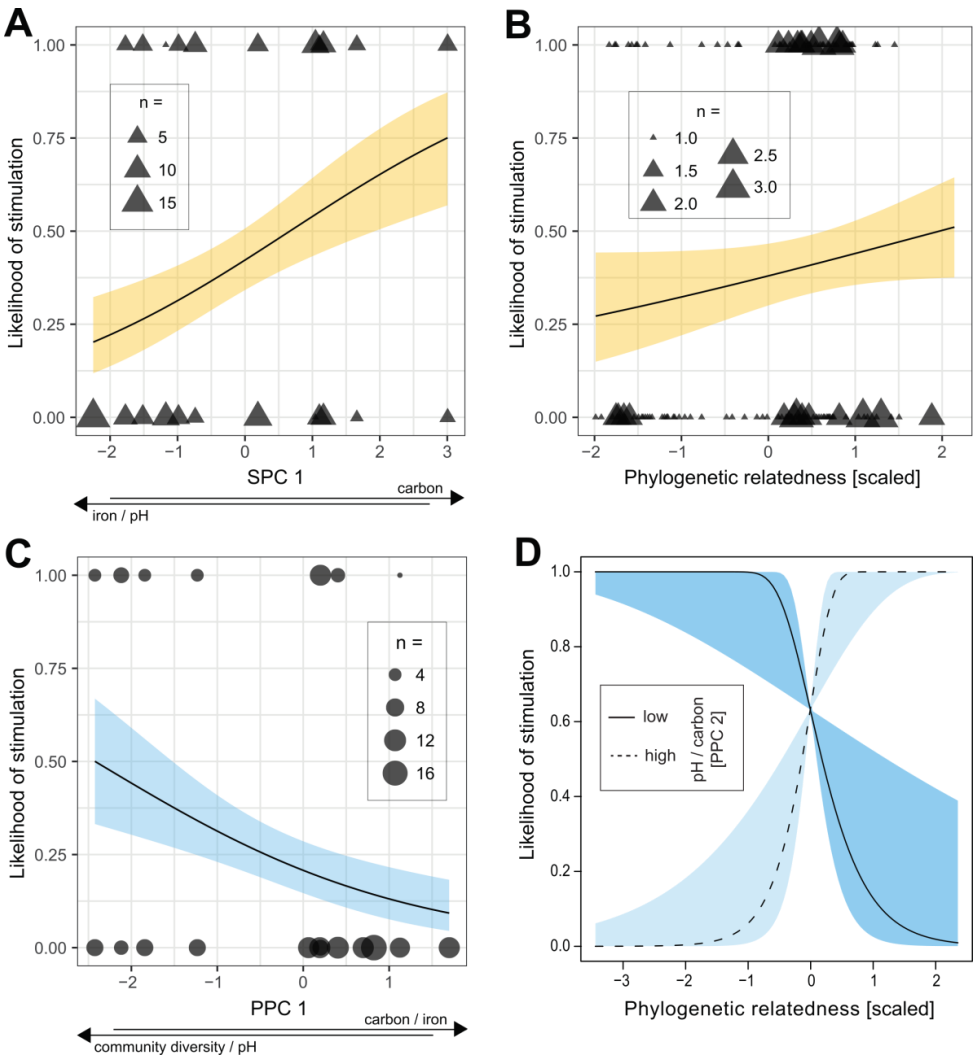


Figure 4. Correlations between parameters characterizing pyoverdine-mediated social interactions, environmental variables, and phylogenetic relatedness between strains. For soil non-producers, the likelihood to be stimulated by supernatants containing pyoverdine from a producer correlated positively with SPC1, i.e. it increased with increased carbon concentration, but reduced levels of pH and iron **(A)**; and phylogenetic relatedness between non-producer and producer based on *rpoD* identity **(B)**. For pond non-producers, the likelihood to be stimulated by producer supernatants correlated negatively with PPC1, i.e. it decreased with decreased pH and community diversity combined with increased carbon and iron concentrations **(C)**. In addition, there was a significant interaction between the *rpoD*-based phylogenetic relatedness between isolates and PPC2, whereby the likelihood of stimulation significantly increased with phylogenetic relatedness for higher values of pH and carbon (high PPC2), but decreased with phylogenetic relatedness for lower values of pH and carbon (low PPC2) **(D)**. Solid lines with shaded areas show significant correlations together with the 95% confidence band for soil (yellow) and pond (blue) communities. Data shown is based on 152 non-producer-supernatant combinations for soil, and 151 non-producer-supernatant combinations for pond.

Table 1. Loadings of the abiotic and biotic environment-defining variables onto the principal components (PCs) for **(A)** soil and **(B)** pond communities.

(A) Soil	**SPC1**	**SPC2**	**SPC3**	**SPC4**
community diversity	-0.1729	**0.9358**	-0.2445	-0.1862
pH	**-0.5396**	0.1584	**0.7812**	0.2711
iron	**-0.5751**	-0.1406	-0.5674	0.5723
carbon	**0.5901**	0.2819	0.090	**0.7512**
explained variance [%]	62.3	25.7	9.9	2.1

(B) Pond	**PPC1**	**PPC2**	**PPC3**	**PPC4**
community diversity	**-0.4892**	-0.3813	**-0.7797**	0.0857
pH	**-0.5161**	**0.5353**	-0.0114	**-0.6686**
iron	**0.5449**	-0.3705	-0.2391	**-0.7132**
carbon	**0.4443**	**0.6564**	-0.5786	0.1924
explained variance [%]	69.5	18.8	9.2	2.5

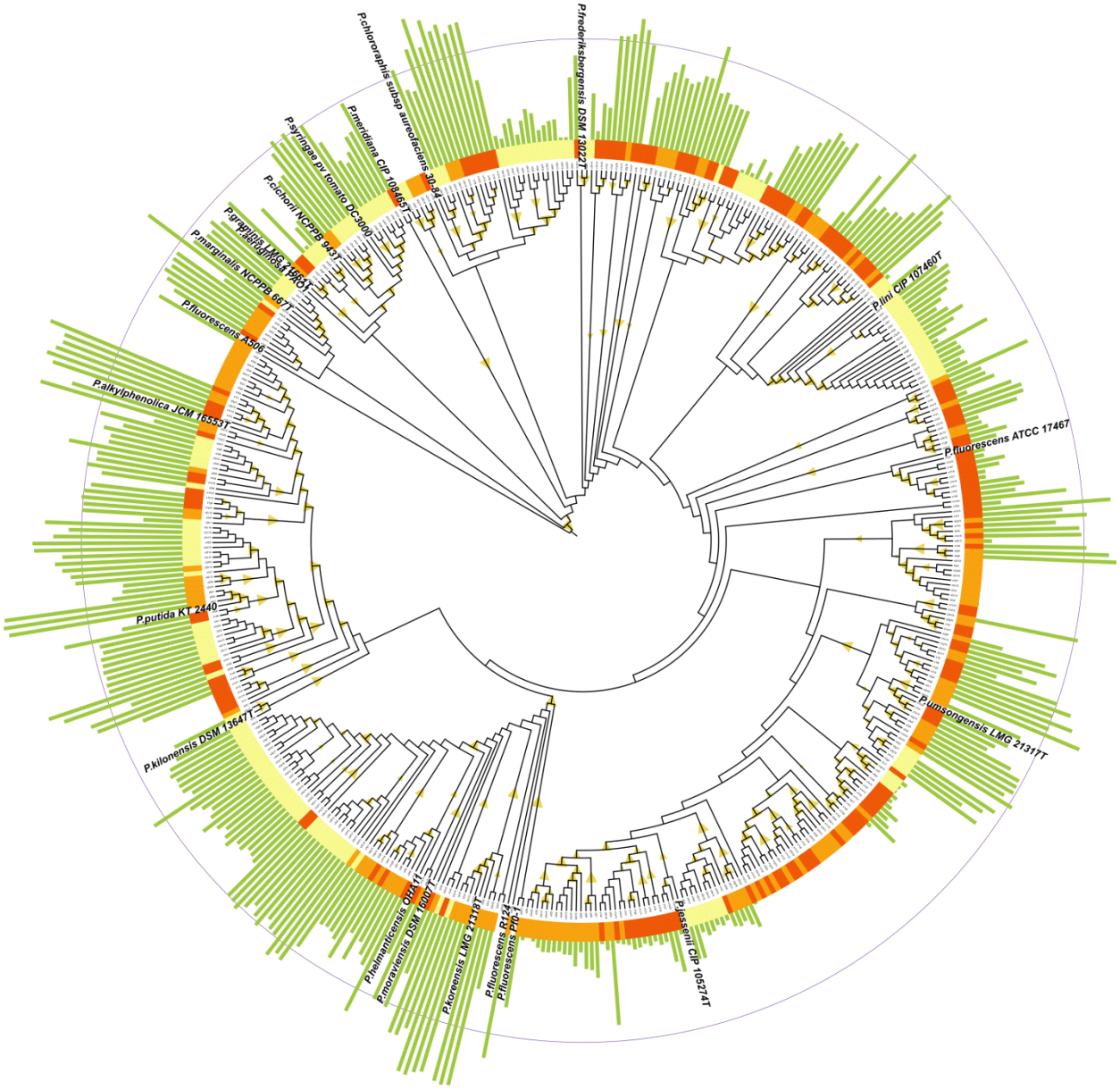
SPC = Soil Principal Components

PPC = Pond Principal Components

Significant loadings are given in bold

3.2 Supporting material

(A) Soil



(B) Pond

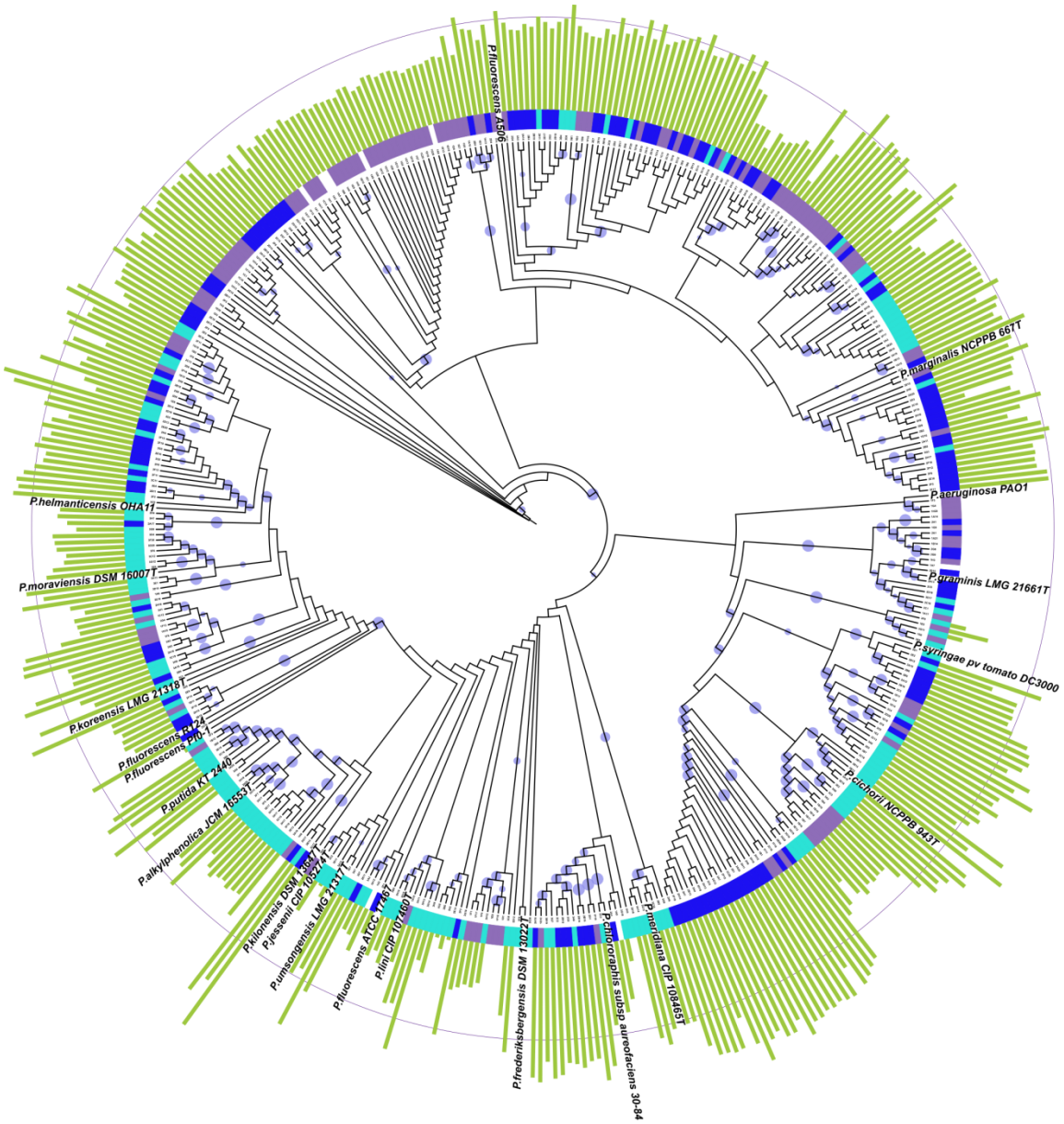


Figure S1. Maximum-likelihood cladograms for soil (A) and pond (B) isolates based on partial *rpoD* sequences. For both habitats, published *rpoD* sequences of 20 members of the *P. fluorescens* lineage and *P. aeruginosa* PAO1 were integrated into the cladograms to demonstrate taxonomic affiliation and diversity of our environmental isolates (as described elsewhere [8]). Yellow triangles and blue circles indicate bootstrap values (50-100%) for branches in the soil (n = 454) and the pond cladogram (n = 433), respectively. Green bars depict pyoverdine production levels of isolates. All pyoverdine values are expressed relative to the production levels of laboratory reference strains (listed in Table S1). The grey circle shows the scaled average pyoverdine production of these references trains. Colour strips around cladograms represent the different locations from which isolates originated.

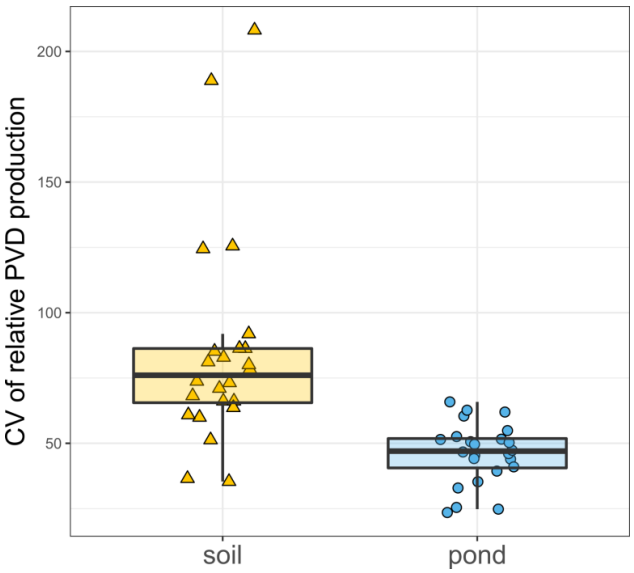


Figure S2. Coefficient of variation for relative pyoverdine (PVD) production is higher among soil than pond communities. Pyoverdine production values were scaled relative to laboratory reference strains listed in Table S1. The coefficients of variation (CV = standard deviation / mean) were calculated separately for each of the 24 soil and 24 pond communities, and based on a total of 462 soil and 468 pond isolates. Box plots show the median (bold line), the 1st and 3rd quartile (box), and the 5th and 95th percentile (whiskers) for each of the two habitat types.

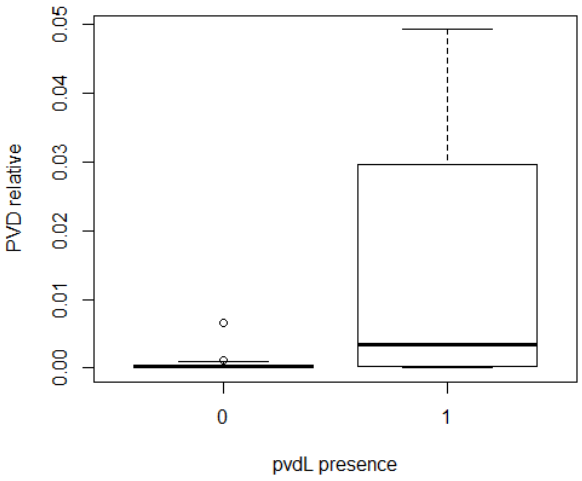


Figure S3. *pvdL*-positive 'non-producers' could produce residual amounts of pyoverdine, compared to *pvdL*-negative isolates that produced no pyoverdine. Presence of *pvdL* gene was evaluated via PCR. Relative pyoverdine production (PVD relative) was calculated by scaling pyoverdine production values relative to laboratory reference strains listed in Table S1. Box plots show the median (bold line), the 1st and 3rd quartile (box), and the 5th and 95th percentile (whiskers).

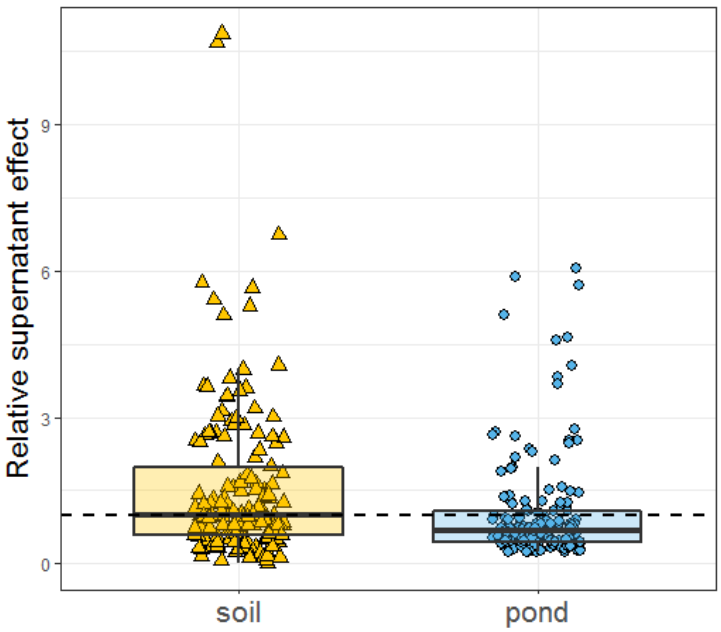


Figure S4. Foreign pyoverdine-containing supernatants can have stimulatory, neutral or inhibitory effects on the growth of non-producers. Pyoverdine-containing supernatants from pyoverdine producers were fed to non-producers from the same community, which grew worse than producers under iron-limited conditions. The growth effect of foreign supernatants on non-producers varied on a continuum from high inhibition to high stimulation. In total, we had 152 soil non-producer-supernatant combinations (yellow triangles), and 151 pond non-producer-supernatant combinations (blue circles). Relative supernatant effect was calculated as the ratio of optical density (OD measured at 600 nm) after 17 h of non-producer's growth with versus without producer supernatant in iron-limited medium (dashed line refers to equal growth under both conditions). Box plots show the median (bold line), the 1st and 3rd quartile (box), and the 5th and 95th percentile (whiskers).

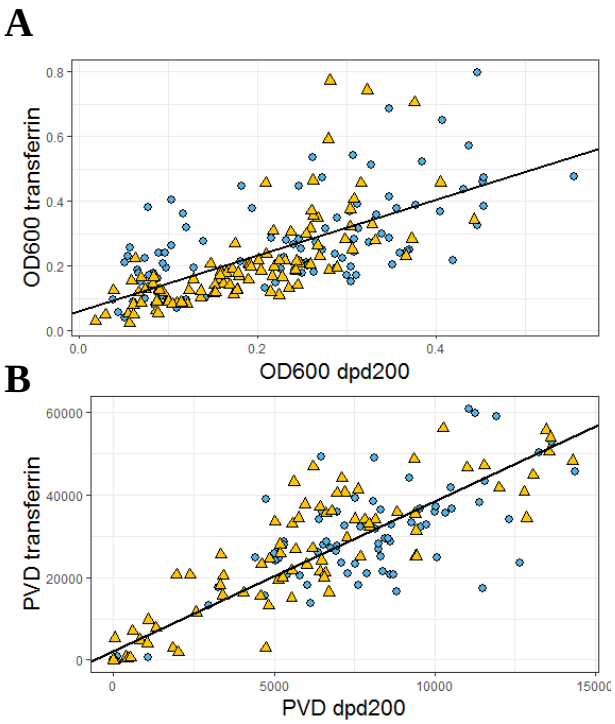


Figure S5. Growth and pyoverdine production profiles correlate positively across two iron-limited media. There were strong positive correlations for growth (OD_{600}) **(A)** and pyoverdine production levels (PVD; relative fluorescence units) **(B),** for isolates grown in iron-limited CAA media where iron was either bound to the synthetic chelator 2,2'-dipyridyl (dpd at 200 μM) or to the natural chelator apo-transferrin (100 μg/ml) (linear models for OD_{600}: $t_{197} = 12.5$, $p < 0.001$, $R^2 = 0.502$; pyoverdine production: $t_{197} = 28.1$, $p < 0.001$, $R^2 = 0.799$). Symbols show means across three replicates for 100 soil isolates (yellow triangles) and 99 pond isolates (blue circles).

Table S1. Reference strains used for growth and pyoverdine measurements.

Strain	Description	Source or reference
P. aureofaciens ATCC13985	wildtype	L. Eberl strain collection, University of Zurich
P. entomophila	wildtype	L. Eberl strain collection, University of Zurich
P. protegens CHA0	wildtype	[1]
P. putida IsoF	wildtype, isolated from tomato rhizosphere	[2]
P. syringae B728a	wildtype	L. Eberl strain collection, University of Zurich
P. aeruginosa PAO1 (ATCC15692)	wildtype; pyoverdine type I	[3,4]
P. aeruginosa 2-164	CF isolate United States; pyoverdine type II	[5,6]
P. aeruginosa ATCC 013	laboratory isolate United States; pyoverdine type III	[5,6]

Table S2. Environmental variables of the soil and pond samples used to isolate pseudomonads.

Community	Habitat type	N° of isolates	Phylogenetic community diversity	pH	Total Fe, µg/g	Total carbon, %	Mean PVDrel*	Non-producers, %
1A	pond	20	0.088	7.6	0.148	0.010	0.596	20
1B	pond	20	0.147	7.6	0.148	0.010	0.729	10
1C	pond	20	0.093	7.7	0.139	0.010	0.881	0
1D	pond	18	0.050	7.7	0.139	0.010	0.765	5.6
1E	pond	20	0.106	7.6	0.107	0.010	0.775	5
1F	pond	20	0.104	7.6	0.107	0.010	0.701	10
1G	pond	20	0.112	7.6	0.090	0.010	0.719	15
1H	pond	18	0.128	7.6	0.090	0.010	0.746	16.7
2A	pond	20	0.085	6.8	0.191	0.007	0.797	10
2B	pond	20	0.100	6.8	0.191	0.007	0.965	0
2C	pond	20	0.118	6.9	0.204	0.007	1.019	10
2D	pond	18	0.157	6.9	0.204	0.007	0.942	11.1
2E	pond	19	0.089	6.8	0.404	0.011	0.914	0
2F	pond	19	0.088	6.8	0.404	0.011	0.863	0
2G	pond	20	0.116	6.9	0.391	0.008	0.827	10
2H	pond	19	0.094	6.9	0.391	0.008	1.074	5.3
3A	pond	20	0.183	8	0.011	0.006	0.855	5
3B	pond	20	0.163	8	0.011	0.006	0.997	5
3C	pond	20	0.191	8	0.010	0.007	0.987	5
3D	pond	20	0.103	8	0.010	0.007	0.947	10
3E	pond	20	0.187	8.1	0.013	0.006	0.714	20
3F	pond	18	0.164	8.1	0.013	0.006	0.669	11.1
3G	pond	20	0.161	8.2	0.012	0.007	0.878	0
3H	pond	19	0.144	8.2	0.012	0.007	0.592	15.8

s1a	soil	19	0.097	7.5	14332	17.3	0.602	15.8
s1b	soil	20	0.096	7.4	14332	17.3	0.578	5
s1c	soil	20	0.050	6.2	6413	35.7	0.166	75
s1d	soil	20	0.093	6.8	6413	35.7	0.294	30
s1e	soil	20	0.077	7.2	8584	31.9	0.650	0
s1f	soil	20	0.067	7.1	8584	31.9	0.582	10
s1g	soil	20	0.127	7	5921	39.6	0.611	20
s1h	soil	20	0.138	7	5921	39.6	0.793	15
s2a	soil	20	0.097	7	14457	29.8	0.365	50
s2b	soil	20	0.167	7.2	14457	29.8	0.747	20
s2c	soil	20	0.093	6.2	7656	44.0	0.775	25
s2d	soil	19	0.086	6.2	7656	44.0	0.648	36.8
s2e	soil	19	0.059	4	7454	45.2	0.168	42.1
s2f	soil	9	0.060	3.6	7454	45.2	0.353	22.2
s2g	soil	18	0.107	7.1	11583	33.5	0.770	22.2
s2h	soil	20	0.169	6.4	11583	33.5	0.679	15
s3a	soil	20	0.072	7.6	18982	6.8	0.564	5
s3b	soil	20	0.132	7.6	18982	6.8	0.543	20
s3c	soil	19	0.108	7.6	14605	5.8	0.919	5.3
s3d	soil	20	0.096	7.6	14605	5.8	0.691	0
s3e	soil	20	0.062	7.5	16290	5.5	0.474	15
s3f	soil	19	0.111	7.5	16290	5.5	0.705	10.5
s3g	soil	20	0.115	7.5	14255	5.7	0.566	5
s3h	soil	20	0.051	7.5	14255	5.7	0.382	10

*PVDrel = relative pyoverdine production, i.e. scaled relative to laboratory reference strains listed in Table S1.

Table S3. Pairwise correlations among variables defining abiotic and biotic environment in **(A)** soil and **(B)** pond habitats.

(A) Soil	community diversity	pH	iron	carbon
community diversity	-	0.1719	0.1343	-0.1099
pH	0.4218	-	**0.8188**	**-0.8954**
iron	0.5317	**0.0010**	-	**-0.8322**
carbon	0.6094	**<0.0001**	**0.0005**	-

(B) Pond	community diversity	pH	iron	carbon
community diversity	-	**0.5261**	**-0.5702**	**-0.7062**
pH	**0.0083**	-	**-0.8744**	**-0.5947**
iron	**0.0036**	**<0.0001**	-	**0.6224**
carbon	**0.0001**	**0.0022**	**0.0012**	-

The (Spearman) correlation coefficients and corresponding p-values are given in the upper right and the lower left quadrant, respectively. Significant values are in bold.

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1. Laville, J. *et al.* Characterization of the *hcnABC* gene cluster encoding hydrogen cyanide synthase and anaerobic regulation by ANR in the strictly aerobic biocontrol agent *Pseudomonas fluorescens* CHA0. *J. Bacteriol.* **180**, 3187-3196 (1998).
2. Steidle, A. *et al.* Identification and characterization of an N-acylhomoserine lactone-dependent quorum-sensing system in *Pseudomonas putida* strain IsoF. *Appl. Environ. Microbiol.* **68**, 6371-6382 (2002).
3. Stover, C. K. *et al.* Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959-964 (2000).
4. Ghysels, B. *et al.* FpvB, an alternative type I ferripyoverdine receptor of *Pseudomonas aeruginosa*. *Microbiology* **150**, 1671-1680 (2004).
5. Smith, E. E., Sims, E. H., Spencer, D. H., Kaul, R. & Olson, M. V. Evidence for diversifying selection at the pyoverdine locus of *Pseudomonas aeruginosa*. *J. Bacteriol.* **187**, 2138-2147 (2005).
6. Jiricny, N. *et al.* Fitness correlates with the extent of cheating in a bacterium *J. Evol. Biol.* **23**, 738-747 (2010).

Chapter 4. Concluding discussion

'The world, like a great iris of an even more gigantic eye, which has also just opened and stretched out to encompass everything, stared back at him.'

~ Ray Bradbury, Dandelion Wine (1957)

4.1 Overview

Bacteria live in complex communities where they engage in different social interactions, such as biofilm formation, foraging and secretion of public goods for nutrient acquisition [49]. These interactions have mainly been studied in well-controlled but contrived laboratory conditions using laboratory strains (except for *Myxococcus xanthus* [57,111]). In this thesis, I aimed to understand how bacteria interact in natural communities. I did this by focusing on the production of pyoverdine, a siderophore secreted by fluorescent pseudomonads. Pyoverdine is a public good (secreted shareable molecule) and its production is a model trait of bacterial cooperation. Pyoverdine and other public goods can be exploited by cheaters: bacteria that do not produce or produce less of the public good but still can benefit by using the public good produced by others [55,56], which reduces the relative fitness of the cooperating producers.

In chapter 2, I presented my project 1 where I explored: (i) whether pyoverdine non-producers are common in nature; (ii) whether they can act as cheaters on producers; and (iii) what the genetic basis of the observed pyoverdine non-production and social interactions are. Previously, it was shown that pyoverdine non-producers can evolve in laboratory settings [112,113] and can act as cheaters [58,92]. Non-producers were also found in natural environments (e.g. human lungs) [76,80], and were assumed to be cheaters as they retained receptors for pyoverdine use. However, direct evidence of bacterial cheating behaviour among environmental isolates was lacking [57].

In my thesis, I could demonstrate that pyoverdine non-producers often co-occur with producers in soil and pond. Some of the non-producers possess highly truncated and completely non-functional pyoverdine biosynthesis locus, while others have an intact and largely inactive locus. Furthermore, the whole genome sequencing of 24 isolates revealed that non-producers (but also the sequenced producers) have multiple pyoverdine receptors, which could allow them to exploit multiple producers. Moreover, we observed not only structural but also functional diversity among pyoverdines produced by different isolates: some pyoverdine types could be exploited by non-producers, while others could not. The latter pyoverdine types could even inhibit the growth of non-producers with incompatible receptors. Importantly, I showed that some of the non-producers indeed could act as cheaters, invading populations of producers secreting a compatible pyoverdine. However, in some cases non-producers could not outcompete producers even if they could exploit their pyoverdine. Thus, non-producers should not be immediately considered as cheaters. All in all, this study suggests that in nature there is selection for both cheating and cheating resistance.

In my project 2 (presented in chapter 3), I examined how environmental factors, which were shown to be important in laboratory conditions, shape siderophore production and social interactions in natural *Pseudomonas* communities. In this project, I investigated whether habitat structuring, pH, total iron and carbon concentrations, and community diversity influence pyoverdine production

levels, the presence/absence of the pyoverdine locus, the ability of isolates to exploit heterologous pyoverdines and pyoverdine competitiveness for iron (i.e. its inhibitory effect on non-producers). My analyses indicate that these factors are also important determinants of pyoverdine production and its social effects in nature. However, contrary to the well-controlled laboratory conditions, in nature we found these factors to be often positively or negatively correlated with each other. Thus, the environmental factors can have combinatorial effects, and/or underlie trade-offs, with these effects being more important than the effect of a single factor. Furthermore, I observed clear differences between soil and pond. For example, the average level of pyoverdine production was higher among pond isolates, which could be due to a much lower iron concentration in pond than soil, and/or a higher loss of pyoverdine due to diffusion in pond. Moreover, I found more non-producers in soils with predicted higher iron bioavailability and concomitantly higher carbon concentration than in more nutrient-limited soils and ponds. Cheating pyoverdine non-producers were shown to be favoured under high iron limitation [26] and when carbon (building block of pyoverdine) is limited [71,72]. Moreover, pyoverdine production is most required under high iron limitation, while under lower iron limitation a secondary siderophore would be used [28]. Thus, prevalence of soil non-producers in habitats with predicted high iron bioavailability and concomitantly higher carbon concentration suggests that these soil non-producers were favoured because of pyoverdine disuse, and not cheating, in the context of iron acquisition. Interestingly, non-producers were not common in pond, contrary to what we would expect based on laboratory experiments, which showed that higher levels of mixing in less structured environments increase the chance of cheating non-producers to benefit from pyoverdine of others and invade population of producers [61]. This observation suggests that cheating pyoverdine non-producers are not common in ponds. Yet, it could be that cheating is prevalent in ponds, but as a facultative strategy among producers: it is beneficial to exploit others but too risky to be a complete non-producer in a nutrient poor, dilute and mixed environment like pond.

Overall, my two projects together suggest that in nature pyoverdine production can be lost/absent due to cheating behaviour and due to high iron availability. Moreover, despite the possibility to exploit producers, cheating non-producers seem to be kept at bay, as suggested by their low numbers in habitats with predicted high iron limitation, and presence of isolates producing non-exploitable pyoverdine. In the next section, I will discuss the findings of my two projects in a wider context. I will also explore how studying sociality of siderophore production can be useful for applied microbiologists.

4.2 What does the ability or inability to use heterologous siderophores mean?

4.2.1 Why does siderophore exploitation not necessarily lead to cheating?

The ability to use heterologous siderophores, also called 'siderophore piracy' is widespread among bacteria [19]. It is expected to confer a selective advantage in the competition for iron, for both non-producers [70] and siderophore producers [32]. Although non-producers, able to use heterologous siderophores, are often called 'cheaters', I could show in my project 1 that such non-producers are not necessarily able to invade populations of producers. Why could that be? One possible explanation is that such siderophores might be partially private (i.e. kept close to the cells via hydrophobic parts) [62,64]. Alternatively, it could be that producers benefit from something produced by the non-producers and even engage in a mutualistic interaction [85,114]. Moreover, receptors of non-producers might have lower affinity for the heterologous siderophores than producers. As non-producers can have multiple pyoverdine receptors or less-specific receptors allowing acquisition of different pyoverdines, such 'take a bit from everybody' strategy might be enough to sustain them in iron-limited conditions, even if receptors do not have a very good affinity for heterologous siderophores. I predict this to be a more efficient strategy in less-structured environments like ponds, than soils. This is because pond isolates, compared to those living in the more structured environment like soil, should more frequently encounter multiple different pyoverdine types and producers, which could promote receptor diversification and/or acquisition of new receptors, even from non-relatives, via horizontal gene transfer. In line with this prediction, pond non-producers were often stimulated by pyoverdine-containing supernatant of phylogenetically distant producers (project 1 and 2). In contrast, soil non-producers were predominantly stimulated by pyoverdine-containing supernatants of producers closely related to them. The latter pattern could be explained by non-producers evolving de novo from producers, thus, they inherently possess the matching receptor to exploit pyoverdine of the producer they originated from, and that they stay in the vicinity of the producer because soil is a structured environment.

4.2.2 The different roles of heterologous siderophore receptors

Next, I address the question of why bacteria retain heterologous siderophore receptors even when they possess their own siderophore or when siderophores are not need for iron acquisition. My results together with other studies suggest that there are at least three mutually non-exclusive explanations. First, receptors for heterologous siderophores might allow facultative cheating: bacteria rely on their own siderophore when growing alone, but switch to exploiting heterologous siderophores when other producers are nearby [32]. Results of the whole genome sequencing (project 1) support the possibility of such facultative strategy, as all the sequenced isolates (even producers) possess multiple pyoverdine receptors.

Second, it was suggested that the presence of heterologous receptors could help to invade new niches occupied by siderophore producers [23]. However, my data from soil communities is not in line with this hypothesis, because soil pseudomonads were mostly stimulated by pyoverdine-containing supernatant of close relatives, which are likely to share the same niche. Thus, soil non-producers do not seem to be good invaders of new niches likely occupied by less related strains. This might be not surprising given the high spatial structure of soil and limited dispersal opportunities. In contrast, this hypothesis might be true for pond non-producers, which seem to use pyoverdines also of distantly related producers and should have more opportunities to disperse, as ponds are less structured than soils. Moreover, the presence of a receptor might help to invade new niches irrespective of the environmental structuring, if it allows the uptake of a siderophore that is, contrary to pyoverdine, not diverse between species (e.g. vibrioferrin of *Vibrio* [70,115]).

The third explanation for the presence of heterologous siderophore receptors is that it offers protection from inhibition by siderophores produced by others [24]. The secreted siderophores can create local iron limitation for the competitors, and like that inhibit their growth. Thus, the presence of compatible receptors allows to counteract such inhibition. This is supported by my observation (project 1) that in pairwise competition assays with producers secreting an incompatible pyoverdine, non-producers were often not detectable anymore after 48 hours. In contrast, non-producers were always maintained in the community when growing with producers of an exploitable pyoverdine, even if the non-producers were unable to outcompete them. Moreover, in my project 2 I observed that many non-producers from soils with increased iron bioavailability (where we expect pyoverdine to be not needed for iron acquisition) were stimulated by pyoverdine-containing supernatant of other community members, suggesting presence of compatible pyoverdine receptor(s). Although abundant in their communities, these non-producers always co-occurred with producers. These results could suggest that ability to use heterologous pyoverdines is useful in counteracting inhibition by producers even in environments where iron availability can be relatively high.

4.2.3 Changing pyoverdine structure as a mechanism to resist cheating

Previous studies have suggested that pyoverdine diversity (more than 100 different pyoverdines are known [104]) is the result of an evolutionary arms race between producers and non-producers, whereby producers evolve new variants of pyoverdine to escape cheating [100,105]. This hypothesis is supported by the results of my project 1, where I could show that non-producers co-occur with two types of producers: producing a pyoverdine that is exploitable or not exploitable by the non-producer. Moreover, in my project 2, I found that the likelihood of stimulation of non-producers by pyoverdine-containing supernatant varied across communities. This suggests that pyoverdine exploitability can vary with properties of the habitats. We expect diversifying selection on

pyoverdine to be high when iron is limited, and even more when additionally carbon (building block of pyoverdine) is limited, as in these conditions cost of pyoverdine production is high [26,71,72], thus cheating would be especially harmful for producers. This is indeed compatible with my results for soil: I found that non-producers from soils with expected highest iron limitation (highest pH, although lower total iron concentration), and concomitantly lower carbon concentration than in other soils, were less likely to be stimulated by pyoverdine-containing supernatants from other community members, than non-producers from habitats with predicted higher iron availability and higher carbon concentration (project 2). Furthermore, pyoverdine-containing supernatants of producers from these habitats tend to be more inhibitory towards non-producers. This further suggests that competition for iron is fierce in these habitats and cost of pyoverdine production is high, selecting for pyoverdines that are less exploitable and even inhibitory towards competitors. However, the inability to use heterologous siderophores, especially in aquatic systems, could also be related to the mixing and openness of the environment (i.e. the introduction of new strains from different environments such as surrounding soils, and via animals or plants), leading to the situation where unrelated (including at the pyoverdine locus) strains temporary co-occur.

It is worth mentioning that in nature, contrary to laboratory conditions, higher carbon concentration might lead to lower cost of pyoverdine production not only because carbon is a building block for pyoverdine [71,72], but because it very likely corresponds to organic matter, like fulvic or humic acid. Organic matter was shown to solubilise iron and potentially increase its bioavailability, especially for siderophore producers, in both soil and aquatic environments [9,11,12,116]. Additionally, it was shown to adsorb siderophores preventing their loss in soil [117], thus, reducing cost of siderophore production. Moreover, in aquatic systems such organic matter can introduce environmental structuring by serving as a habitat for bacteria [70,118], which could allow relatives to stay close to each other, more efficiently share pyoverdine and be less exposed to cheaters.

Interestingly and contrary to soil, pond habitats with predicted highest cost of pyoverdine production and most fierce competition for iron (not only most limited in iron, but also with lower carbon concentration, and higher community diversity suggesting presence of more competitors) seem to favour non-producers that can use heterologous pyoverdines to overcome iron limitation, compared to other pond habitats (project 2). Why does pyoverdine exploitation seem to be favoured over cheating resistance in the ponds with predicted highest cost of pyoverdine production, contrary to the soils with predicted highest cost of pyoverdine production? I propose three related explanations that could be based on differences in: (i) the degree of environmental structuring; (ii) the ability/inability to exploit multiple producers; and (iii) the extent of niche overlap. Being less structured, ponds allow more mixing of strains and their public goods. Therefore, changing pyoverdine might be a useless strategy for pond producers because environmental mixing would soon bring them in touch with other potential cheaters. In contrast,

soils are more structured and less open systems (less migration), thus, producers can more easily avoid being surrounded by de novo or invading cheaters. Furthermore, and as already discussed before, pond isolates should have more opportunities to acquire novel receptors via horizontal gene transfer or experience selection to evolve less specific receptors, allowing pyoverdine acquisition from multiple and even phylogenetically distantly related strains. We could speculate that the already mentioned 'take a bit from everybody' strategy, likely employed by pond isolates, might be well-tolerated by producers which would not lose much pyoverdine in this case. Moreover, less-related producers might tolerate exploitation more than closely related producers, because they are likely to occupy a different niche, and thus compete less [74]. If exploitation of non-related producers can indeed be better tolerated than exploitation of related species, why do soil non-producers not adapt to exploit non-relatives (both of my projects indicate that soil non-producer tend to use pyoverdines of closely related strains)? This might be difficult, as soil is a structured environment (except for waterlogged soils), thus, non-producers might not have many opportunities to be exposed and adapt to exploit less-related producers.

4.3. Diversity of microbial communities

4.3.1 Factors promoting and stabilizing diversification of bacterial communities

I found environmental pseudomonads to be highly diverse, including diversity at the phylogenetic, the phenotypic and the interaction level. In general, bacterial communities show remarkable diversity [119]. This degree of diversity might be surprising, as bacteria engage in different competitive interactions, which should ultimately lead to the extinction of less fit phenotypes, when species compete for the same resources [120]. Different species are predicted to co-exist only if intra-specific competition is stronger than inter-specific competition [121]. In accordance with this theory, different mechanisms were suggested that could promote or maintain the huge bacterial diversity, like spatial heterogeneity or differentiation of the resources used. Spatial heterogeneity, for example, could allow weaker phenotypes to hide from stronger competitors and/or differentiate to use an alternative food source than the competitor. Such differentiation would eliminate the inter-specific competition, but not the intra-specific competition occurring between clonal bacteria using the same resources. Indeed, such mechanisms were observed in fast diversifying bacterial populations, which lead to niche partitioning, resulting in the co-existence of different bacterial strains in a structured environment [122]. Living in a biofilm (a layer of surface-attached bacterial communities) also can easily foster diversification and co-existence of different bacteria: because of structuring different chemicals (nutrients and extracellular products) are limited in their diffusion, resulting in chemical gradients that can generate different microniches. This, in turn, can lead to bacterial diversification leading to differential use of resources, thus, co-existence of different bacteria [53]. Heterogeneity of the environment promoting community diversity could also

explain the higher variation in pyoverdine production that I observed within soil communities, compared to the pond communities. Although less structured than soils, ponds, however, can also be a relatively structured environment [118].

The results of my projects suggest that pyoverdine-mediated cooperation, exploitation and competition could also significantly contribute to biodiversity of bacterial communities in nature. In my project 1, I found exploitable pyoverdine producers coexisting with exploiting non-producers and non-exploitable producers of a different pyoverdine. As already discussed before, evolution of such non-exploitable pyoverdine producers was hypothesized to be promoted by the presence of a cheater [100,105], which would drive pyoverdine diversification as a mean to avoid being exploited. But how could this diversity be maintained? One possibility is that the observed diversity in pyoverdine use and inhibition is favoured because it leads to non-hierarchical competitive interactions between strains. Such interactions among bacteria were predicted and shown in laboratory studies to help to maintain community diversity in structured [123] and non-structured environments [124,125]. An example of such interactions would be a community of three competitors engaged in interactions that can be compared to the game 'rock–paper–scissors': scissors cut paper, paper covers rock and rock destroys scissors, whereas simultaneous appearance of all the three figures lead to parity. Such interactions were shown, for example, to allow cheated siderophore producer, cheater and non-cheatable another siderophore producer to co-exist even in non-structured environments [125]. Similarly to the dynamics described by Inglis et al. (2016) [125], diversity in antibiotic production was shown to be maintained even in a well-mixed environment: the inhibitory effect of an antibiotic producing strain on a sensitive strain is counteracted by an antibiotic-degrading strain [126].

4.3.2 'Key-lock' diversity

Next, I want to discuss whether the pyoverdine-receptor diversity is a curiosity of pyoverdine production/uptake system or whether we can find analogies in other systems.

Pyoverdine ('key') and its receptor ('lock') were shown to be under diversifying selection in *P. aeruginosa* [100]. This seems also to be true for other *Pseudomonas* species, where a huge pyoverdine diversity was observed [99]. The high pyoverdine and receptor diversity is intriguing. But do we find a similar diversity in other microbial public good systems? Indeed, the high 'key-lock' [110] diversity was also observed for genes involved in biosynthesis of quorum signalling molecules and their corresponding receptor in natural populations of *Bacillus* spp. [127]. Quorum sensing is a cell-cell signalling mechanism which allows bacteria to adjust gene expression, including production of different public goods, based on cell density. This 'key-lock' diversity was suggested to serve as a kin recognition mechanism, promoting interaction among relatives from the same ecological population [110,128]. A clear example of kin recognition is the formation of boundary lines between

swarming colonies of different strains of the same species, including *Proteus mirabilis* [129], or *M. xanthus* [66], because colonies of non-kin do not merge. However, in quorum sensing we do not have a typical example of kin recognition: kin recognition allows to start cooperating with the kin, but it does not prevent exploitation of public goods by non-kin with incompatible quorum sensing system present in the same population [130]. The diversity of bacteriocins and contact-dependant inhibitory toxins were also suggested to serve as a kin recognition mechanism [128]. However, this explanation seems to be unlikely for the diversity of pyoverdine and its receptor, given the frequent presence of multiple and/or less specific receptors in different species.

4.3.3 Multiplicity of public-good interactions in natural communities

While in my projects I focused on a single trait, we know that bacteria can secrete multiple goods at the same time. This raises the question how social interactions change in a multi-public goods situation, which is most probably the case in natural communities. For example, pyoverdine non-producers might provide some metabolites that are beneficial for producers whose pyoverdine they can use, and thus engage in a mutualistic interaction [85,114]. This would be in analogy to the obligate cross-feeding interactions that are wide-spread among bacteria and were shown to foster community diversity [131]. On the other hand, pyoverdine non-producer might be 'super-cheats' exploiting multiple public goods of others [132]. Additionally, loss of production of one public good might have pleiotropic effects, for example, upregulation of another public good, which stabilizes cooperation [133]. It remains to be further explored how production or non-production of different public goods are related to each other, how the battle between competition and cooperation is managed in nature, and what evolutionary dynamics are operating there in shaping the highly diverse bacterial communities. My big collection of soil and pond isolates offers a great opportunity to unravel these questions.

4.4. How can siderophore producers and our knowledge on social evolution of siderophore production be applied?

In this section I want to discuss how our knowledge on environmental determinants of siderophore production and its sociality can be applied to: (i) control plant pathogens; (ii) detoxify soils polluted with heavy metals; and (iii) fight human pathogens.

Pseudomonads and their siderophores have an enormous application potential. For example, many pseudomonads are known as plant growth-promoting bacteria, because their siderophores make iron more bioavailable for plants, which can use reductases to strip off iron or directly take up bacterial siderophores [134,135]. As iron is essential for most bacteria, pyoverdine producers can be used as bio-control agents inhibiting different plant, fish and other pathogens, by sequestering iron which is usually very limited in nature [136,137]. Moreover, different siderophores are able to bind

efficiently not only iron, but also other heavy metals, like Cr^{3+}, Al^{3+}, Cu^{2+} [138]. Although required for different cellular processes, metals typically become toxic at high bioavailable concentrations, especially because of the generation of reactive oxygen species [3,139]. By binding different heavy metals, siderophores can prevent the entry of toxic metals into the cells. Thus, they were proposed to be used as environment-friendly bioremediation agents [16].

It seems attractive for pathogen control and bioremediation to use siderophore producers instead of purified siderophores, as bacteria can multiply and react to their environment, for example, by upregulating their siderophore and potentially different toxin production when the pathogen is present. Moreover, direct application of siderophores would be costlier and require efficient methods of their large-scale purification. However, the danger of using siderophore producers lies, apart from their unpredictable long-term effect on the environment, in the spread of siderophore non-producers. This could lead to the collapse of the cooperative pathogen control or heavy-metal bioremediation by siderophore producers. Thus, it is very important to understand the factors preventing spread of siderophore non-producers, and fostering siderophore production.

4.4.1 Fighting plant pathogens

What could be advised when using siderophore producers to inhibit plant pathogens? Plant surfaces are structured environments, so they should restrict spread of cheating non-producers, unless the structure is destroyed, for example by the rain or watering. If siderophore producers are protecting the above-ground parts of the plant, it could be beneficial to protect plants from the rain and water them at the base of the plant, like that preserving communities of producers undisturbed. It could be advised to apply pseudomonads in the evening or night and keep plants away from direct sunlight, as pyoverdine is light sensitive [140], so its production would be costlier during the day when it can be degraded by direct sunlight. Moreover, sunlight promotes reduction of ferric iron to the soluble ferrous iron (at least in aquatic systems [4]), thus, pyoverdine might be not produced because of high enough iron bioavailability. As pyoverdine production is less costly when carbon concentration is higher [71,72], which disfavours cheating non-producers, it could be beneficial to spray plants with some carbon source prior to the application of the beneficial pseudomonads. In support of usefulness of such treatment, addition of carbon was in general shown to promote antagonistic effect of pseudomonads under iron limitation in soil, probably because it promoted higher siderophore production, as shown in vitro [141]. Most importantly, one should be sure that the plant parts where the beneficial siderophore-producing bacterium is applied are enough iron-limited to promote siderophore production. On the other hand, some toxins of beneficial bacteria were shown to be produced only when iron is replete [142]. Thus, if iron status on the plant surface is not clear or mixed, one could also use a beneficial bacterium which secretes a potent siderophore when iron is limited, and a toxin when iron is replete, both killing the pathogen of interest.

4.4.2 Heavy-metal detoxification

Compared to the siderophore cooperation for iron acquisition, siderophore-based heavy metal bioremediation of soils seems to be even more prone to the spread of cheaters [93]. Siderophore-based detoxification of heavy metals is beneficial not only for closely related strain but also other community members, thus increasing the number of potential cheaters. Such detoxification could potentially lead to the evolution of an obligate dependency, as described by the 'Black Queen hypothesis' [84] theory. The theory predicts such dependencies to evolve when some community members lose an essential function because they have a continuous access to 'leaky' (partially available to others) functions performed by others, resulting in their genome reduction. As siderophore production might be essential in order to survive in the contaminated environment, we can expect siderophore producers and cheaters to be under negative frequency-dependent selection [143], thus, siderophore producers would never get completely extinct. However, for bioremediation to be efficient, the spread of non-producers should be prevented as much as possible.

It is well established that total metal concentration is not an indication of its availability in soil and thus, of its toxicity [8,13]. pH is the main factor affecting iron (and other metal) bioavailability in nature: metal bioavailability tends to increase with decreasing pH both in soil and aquatic environments [4,8,9]. Moreover, iron complexing organic compounds (e.g. humic and fulvic acids) can solubilise iron in soil [10], and other environments, and potentially increase bioavailability of iron [9,11,12,116]. In my project 2, I observed most pyoverdine non-producers in soils that had at the same time low pH and high carbon concentration (organic matter). Even if these habitats had relatively lower total iron concentration (compared to the high-pH, lower-carbon soils), it is very probable that in these soils iron (and other metal) availability was high. These results suggest an additional caveat in using siderophore producers for bioremediation – the high iron bioavailability in soil favours spread of siderophore non-producers because siderophores are not required for iron acquisition. In line with this prediction, Hesse et al. (2017) [144] also observed that siderophore (not only pyoverdine) non-producers were favoured in low-pH soils even if such soils concomitantly had relatively lower total iron concentration. Siderophores might still be required for heavy metal detoxification in such soils, but a small fraction of producers in a community might be sufficient to allow survival of the community members. The situation might be also aggravated if soils contaminated with heavy metal get waterlogged, as iron bioavailability in such soils is high [7,13] and environment becomes less structured, which favours cheating siderophore non-producers [61].

Based on my results and many other studies on iron bioavailabilty and siderophore sociality, what could be advised for those using siderophore producers for bioremediation? The main step would be to make soil more alkaline, which would reduce iron (and other metal) solubility, and thus favour siderophore producers. Moreover, iron solubility can also be reduced by increasing access of oxygen, for example, by aerating soil. Next, if soil is very wet it would be beneficial to dry it,

which would not only reduce iron biovailability but also increase structuring of environment, which should favour siderophore producers. Finally, as metal availability tends to be lower at lower temperatures [13], it might be better to use siderophore producers in the evening and colder season.

4.4.3 Fighting human pathogens

Siderophore producers and our knowledge on sociality of siderophore production can also be used in medicine. For example, sideromycins (conjugates of siderophores and antibiotics, natural or synthetic) can be used as a 'Trojan Horse strategy' to kill bacteria using siderophore receptors for the entry [145]. However, we should be sure that iron is indeed limited in the targeted infected part of the body, and thus, loss of pyoverdine receptor is not selected for. For example, cystic fibrosis lungs after some time of chronic bacterial infection very probably are both iron- and carbon-replete because of the tissue damage [82]. Moreover, one should be sure that alternative efficient iron acquisition systems are not present in the bacterium, for example, bacteria can use host iron-chelating molecules instead of producing their own siderophore [86]. Otherwise, use of sideromycins and approaches targeting siderophore production (like addition of Ga^{3+} that irreversibly binds pyoverdine [146]; introduction of cheating less virulent siderophore non-producers [96]) would be useless. It is crucial to use more realistic conditions when testing the possibility of harnessing siderophore production and/or uptake to treat infections [82]. For example, instead of too contrived testing in artificial simple medium, pig lungs could be used.

Interestingly, among the 930 isolated soil and pond pseudomonads, I found no *P. aeruginosa*, the opportunistic human pathogen commonly infecting lungs of cystic fibrosis and immunocompromised patients, and also burns. This finding suggests that *P. aeruginosa* is not successful in such habitats, although ubiquitous on humans. The same observation was made by Chatterjee et al. (2017) [147]: they found no *P. aeruginosa* among their 330 pseudomonads isolated from soil and freshwater environments. This observation prompted them to explore the potential of these natural isolates to inhibit *P. aeruginosa*. Indeed, they found that different isolates efficiently inhibit *P. aeruginosa*. Like me, they also observed that pond isolates are more inhibitory towards other pseudomonads, than soil isolates, although they did not use iron-limited conditions like I did. These isolates produce different metabolites that inhibit *P. aeruginosa* and potentially different other pathogens. Thus, they represent a great potential for the discovery of novel antimicrobials. Would I suggest using such natural isolates to inhibit *P. aeruginosa* in infections? Yes, I would use them, especially the pond isolates, as they are more inhibitory. However, I would not use pyoverdine of these isolates to fight *P. aeruginosa*, even if they efficiently inhibit *P. aeruginosa*. This is because pyoverdine receptor and pyoverdine, as mentioned before, are under diversifying selection [100], thus, I expect resistance to come up fast. I would, however, use these pyoverdines to inhibit other non-*Pseudomonas* bacteria that have less efficient iron acquisition strategies and do

not have a pyoverdine receptor. To my knowledge, only pseudomonads have a pyoverdine receptor, suggesting that its inter-genera horizontal gene transfer is not occurring. Therefore, in iron-limited conditions use of pyoverdines against other bacteria might be an efficient evolutionarily robust strategy.

All in all, my collection of soil and pond pseudomonads already has revealed many exciting aspects of pyoverdine production and sociality in nature. Many more secrets of nature are waiting to be brought to light using this collection. And many different applications are waiting to contribute to human well-being.

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This list of references includes the general introduction and the concluding discussion. References from individual projects are listed at the end of their respective sections.

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